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STRUCTURAL STUDIES OF A NOVEL EXOPOLYSACCHARIDE PRODUCED BY A MUTANT OF *Rhizobium meliloti* STRAIN Rm1021

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ABSTRACT

The structure of a novel expolysaccharide obtained from a mutant of *Rhizobium meliloti* strain Rm1021 was elucidated by a combination of enzymic, chemical, and spectroscopic methods. The polysaccharide is composed of a disaccharide repeating-unit, β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3), having a 6-*O*-acetyl group attached to most D-glucose residues and a 4,6-*O*-(1-carboxyethylidene) group attached to every D-galactose residue.

INTRODUCTION

Bacteria of the genus *Rhizobium* fix nitrogen in symbiotic association with leguminous plants. In the course of this association, the bacteria induce the formation of nodules on the plant roots, which they then invade through tubes called infection threads. Upon entering the nodule, the bacteria differentiate into the bacteroid form, and begin to fix nitrogen. Effective nodulation of leguminous plants by many *Rhizobium* species requires extracellular polysaccharides which are produced by these bacteria[1–4]. Mutants of *R. meliloti* strain Rm1021 have been isolated that are unable to produce an acidic exopolysaccharide, designated EPS I, but do induce the formation of nodules on alfalfa *(Medicago sativa)* roots. Interestingly, however, these structures are not invaded, resulting in empty nodules that do not fix nitrogen[1,5].

Recently, a spontaneous mutant of strain Rm1021 has been isolated[6] that produces a second expolysaccharide identified as EPS II. Derivatives of this strain that synthesize EPS II, but not EPS I, induce and invade nodules, and fix nitrogen. This suggests that EPS II can perform the roles in nodule development which is normally performed by EPS I. In an effort to determine which of the structural features of EPS II are important for these specific symbiotic functions, we have determined the structure of EPS II, and compared it with the known structure[7] of EPS I.

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RESULTS

^{1}H-N.m.r. spectroscopy of EPS II. — The ^{1}H-n.m.r. spectrum of EPS II provided signals which were assigned to the methyl protons of a 1-carboxyethylidene and an acetyl group, and anomeric protons of the α- and β-D-pyranosyl sugar linkages. The ^{1}H-n.m.r. spectrum of EPS II resembled that of EPS I in having singlets at δ 1.46 and 2.15. These signals correspond to the methyl protons of the 1-carboxyethylidene and acetyl groups, respectively. The peak areas of these signals indicated a 1.0:0.7 ratio. Signals at δ 4.75 and 5.4 were assigned to the anomeric protons of β-D-pyranosyl and α-D-pyranosyl residues. Owing to the viscosity of the solution, the coupling constant of the α-D-anomeric proton (δ 5.4) was not extractable from the ^{1}H-n.m.r. spectrum. The integrated areas for the anomeric protons were approximately equal.

Composition of EPS II. — Composition of EPS II was studied by g.l.c.–m.s. using the alditol acetate derivatives. Glucose and galactose are the only sugars and the g.l.c. peak areas indicated an equal concentration. These results suggest that EPS II is composed of disaccharide repeating-units.

F.a.b.-m.s. analysis of fragments of EPS II obtained by glycosidase treatment. — The EPS II polymer was too large for direct analysis by f.ab.-m.s. Thus, to generate smaller fragments, the sample was treated with a crude glycosidase preparation obtained from lyzates of the *R. meliloti* transducing phage ϕM12. The resulting EPS II fragments were partially purified by gel-filtration on a P-4 column which provided a mixture of tetra- an hexa-saccharides. These products were derivatized at the reducing terminal residue with pyridinylamine[8] and analyzed by positive f.a.b.-m.s. This latter step enhances ion abundance and directs glycosidic fragmentation from the terminally located, charged pyridinylamine group. In the mass spectrum of the glycosidase-treated polymer (Fig. 1), the ion at *m/z* 967 could be accounted for by considering a pyridinylamine (93 Da)-derivatized tetrasacchar-

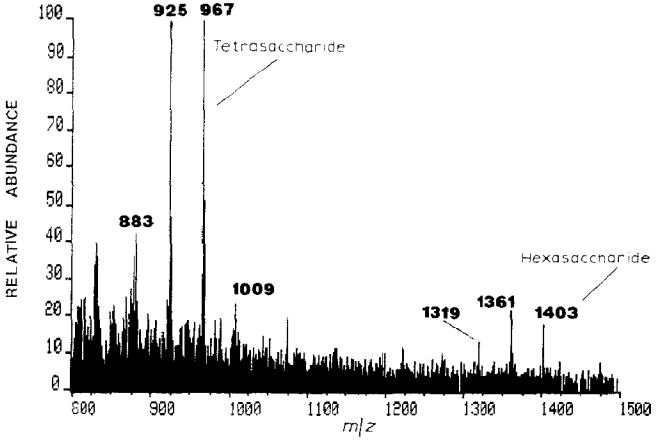


Fig. 1. Positive, fast-atom bombardment-mass spectrometry (f.a.b.-m.s.) of glycosidase-treated EPS II.

ide (162 × 4 = 648) containing two acetyl (84 Da) groups, two 1-carboxyethylidene (140 Da) groups, and a terminal hydrogen atom. The ion at *m/z* 1403 corresponded to a hexasaccharide conjugated with three acetyl and 1-carboxyethylidene groups. Each of these ions are paired with an ion of 42 Da lower in mass, which would correspond to some of the dimers showing an absence of acetylation, or a fragment-ion due to ketene ($CH_2C{=}O$) elimination, a loss very characteristic of acetylated oligomers, or both. Because the mass difference between these two paired ions (1403 − 967 = 436) corresponds to two hexose units and a single acetyl and 1-carboxyethylidene residue, it is probable that most monomer units carry a single conjugated group. These data, along with the composition analysis (glucose to galactose ratio of 1:1) indicated the major repeating-unit of EPS II to be (glucose, galactose, 1-carboxyethylidene, acetyl)$_n$.

Determination of linkages of EPS II. — Linkages in EPS II were determined by g.l.c.–m.s. after sample permethylation, hydrolysis, reduction, and peracetylation[9]. They indicated a 3-substituted D-glucosyl and a 3,4,6-trisubstituted D-galactosyl residue (Table I). Because substitution by a 1-carboxyethylidene group would block two hexose oxygen atoms, this group is attached to a D-galactose units and the third oxygen atom is glycosidically linked to the adjacent D-glucose unit. These results established a disaccharide repeating-unit composed a 4,6-*O*-(1-carboxyethylidene)-D-galactosyl residue linked (1→3) to a D-glucose unit. The alkaline conditions of permethylation causes the hydrolysis of acyl residues and, thus, the location of *O*-acetyl groups in the D-glucose units could not be determined by permethylation.

Determination of oligomer sequence by d.c.i.–m.s. — D.c.i.–m.s. has proven to be a very useful technique for determination of the molecular weight and oligomer sequence of permethylated and peracetylated oligosaccharides[10]. Although the molecular weight of the permethylated EPS II (>20 000) exceeds the workable mass-range of this technique, useful structural information may be obtained by d.c.i.–m.s. with the production of pyrolytic fragments that yield residue molecular weights and partial sequence information. To evaluate this approach, a fraction of the permethylated EPS II sample prepared for linkage analysis was analyzed by d.c.i.–m.s. (see Fig. 2). The sequence of ion fragments (*m/z* 774, 978,

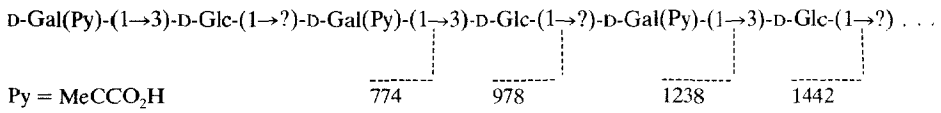
TABLE I

METHYLATION ANALYSIS OF EPS II POLYSACCHARIDE

O-Methyl sugar	*Mol (%)*	
	A[a]	*B*[b]
D-Gal-2-	48	31
D-Glc-2,4-		48
D-Glc-2,4,6-	52	20

[a]Procedure of Hakomori[10]. Procedure of Prehm[12].

1238, 1442, and 1702) can be understood by considering a linear, permethylated hexose polymer having alternating residues conjugated with a single 1-carboxyethylidene group. This hypothesis is consistent with the spectrum obtained earlier (Fig. 1) and the loss of the acyl group during permethylation. Thus, the ion at *m/z* 774 corresponds to a fragment derived from the end of the polysaccharide chain. It consists of three permethylated hexose residues (two of which being conjugated with a 1-carboxyethylidene group), formed by pyrolysis on the reducing side of the glycosidic oxygen atom. The following ions at *m/z* 978, 1238, 1442, 1702, . . . with increments of 204 and 260 Da are additional glycosidic rupture fragments that provide monomer residue molecular weights, and hence, oligosaccharide sequence. These results, combined with the linkage and composition data obtained earlier, and the suggestion that the 1-carboxyethylidene group is conjugated to a galactose residue, suggest structure **1** for EPS II (see Scheme 1).



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Scheme 1. Fragments obtained by d.c.i.–m.s. of permethylated EPS II.

Determination of the location of the acetyl group. — The characterization of the point of linkage of an ester substituent requires alternative procedures of permethylation, as the strong base-catalyzed reactions[11,12] cause acyl hydrolysis. The procedure developed by Prehm[13] avoids strong alkali and retains acyl substituents. Analysis of EPS II with this method again yielded a 3,4,6-tri-*O*-substituted galactose and a new 3,6-di-*O*-substituted glucosyl residue (Table I). A 3-*O*-substituted glucose was also detected with a peak area approximating 25% of that of the disubstituted residue. The latter product was not the result of deacetylation during permethylation, as shown by the study of known acetylated standards. The aforementioned finding are also consistent with the n.m.r. data which showed a 1-carboxyethylidene-to-acetate ratio equal to 1:0.7. The detector responses obtained from g.l.c. were not calibrated with standard compounds and must be considered approximate only. The results established the position of the acetyl group at O-6 and that of the galactosyl residue at O-3 of the glucosyl residue.

Determination of the location of the 1-carboxyethylidene group. — The linkage analysis of EPS II gave three hexitols, *i.e.*, a trisubstituted galactose, and a mono- and di-substituted glucose. Glycosidase-treated EPS II, analyzed by f.a.b.-m.s., provided a tetra- and a hexa-saccharide. These results, in combination with d.c.i.–m.s. and the linkage analysis on the intact polymer, indicated the sequence, Gal(1-carboxyethylidene)→Glc(Ac)→Gal(1-carboxyethylidene)→Glc(Ac). A comparison of the results of permethylation procedures indicated the position of the acetyl group at O-6 of glucose. To identify the location of the 1-carboxyeth-

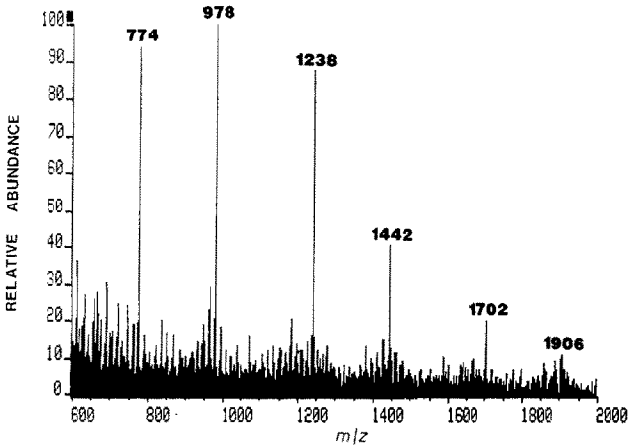
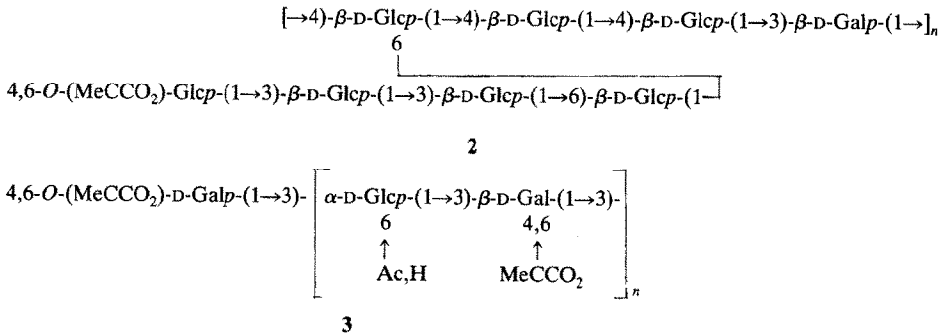


Fig. 2. Positive, direct chemical-ionization–mass spectrometry (d.c.i.–m.s.) of permethylated EPS II. Ammonia was used as the reagent gas (see Scheme 1).



ylidene group on the tri-*O*-substituted galactose, the tetra- and hexa-saccharides, obtained by glycosidase treatment of EPS II, were analyzed by the standard permethylation procedure[10,11]. G.l.c.–m.s. analysis of these products indicated a 4,6-di-*O*-substituted galactose, in addition to the previously identified 3,4,6-tri-*O*-substituted galactose. Since linkage analysis on the intact EPS II produced only a single tri-*O*-substituted galactose, this new di-*O*-substituted product, obtained from glycosidase-treated EPS II, indicated a terminal location for the 4,6-*O*-(1-carboxyethylidene)-D-galactose group.

Determination of anomeric configurations by ^{1}H-n.m.r. spectroscopy. — Integration of the peak areas assigned to the α and β anomeric protons in EPS II showed them to be equal. These signals were assigned to each hexose unit by quantification of the tetrasaccharide spectrum after reduction of the reducing terminal group. The previous results indicated a Gal→Glc→Gal→Glc sequence for the tetrasaccharide. The integrated signals for the reduced tetrasaccharide gave an α-to-β ratio of 2:1 corresponding to the sequence α-D-Gal→β-D-Glc→α-D-Gal→D-Glcol.

DISCUSSION

The structures of EPS I (**2**) and EPS II (**3**) show that the two exopolysaccharides vary considerably in composition, linear sequence, and oligomer branching. EPS II appears to be devoid of branching but is more complex in the attached conjugate groups which modify the majority of hexose residues. The basic unit for this polysaccharide appears to be a β-linked D-Gal→D-Glc disaccharide with each D-galactose residue in the dimer being conjugated with a 1-carboxyethylidene group and ~70% of the D-glucose residues being 6-*O*-acetylated. Closer examination of the f.a.b.-m.s. data suggests that ~5–10% of these dimers may have an additional *O*-acetyl group (see *m/z* 1009, Fig. 1); however, its location must be random because no discrete peaks at that concentration observed in the f.a.b. mass spectra could be observed in the g.l.c. pattern. It is of interest that the 1-carboxyethylidene group is linked to the D-glucose residue in the case of EPS I, and to the D-galactose residue in the case of EPS II. The two exopolysaccharides do share a common structural unit, *i.e.*, the disaccharide β-D-Glc*p*-(1→3)-D-Gal, although this unit is greatly modified and in much higher concentration in EPS II. It is possible that a greater variation occurs in EPS I, since the positions of the *O*-succinyl and *O*-acetyl groups in EPS I have not been determined.

It is known that the structural features of the exopolysaccharides are important for their symbiotic function. Mutants of strain Rm1021 that fail to succinylate EPS I form empty, ineffective nodules[14]. In light of this fact, it is remarkable that the two exopolysaccharides (with as many structural differences as EPS I and EPS II) can replace each other in nodule development. It seems likely that the disaccharide, β-D-Glc*p*-(1→3)-D-Gal, which is the only structural motif that EPS I and EPS II have in common, is required for their symbiotic function. This suggestion is supported by recent work[6] which indicated that some plants can be effectively nodulated by EPS I-producing strains but not by strains that produce EPS II. This effect of exopolysaccharides on the host suggests to us that while general properties such as acidity may be required for exopolysaccharide function, more specific structural features are also involved. The disaccharide unit, β-D-Glc*p*-(1→3)-D-Gal, is common to both EPS I and EPS II, and important for nodulation of *M. sativa*.

Our finding that the β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3) repeating unit in EPS II is heavily modified might be a clue that one of the functions of exopolysaccharides is to help the bacteria avoid plant-defense responses. Kombrink *et al.*[15] have shown that (1→3)-β-D-glucanases are involved in plant-defense responses to pathogens. If the succinyl group in EPS I is linked to the β-D-Glc-(1→3)-D-Gal dimer, this could explain the lack of symbiotic function for ESP II which does not possess succinyl groups. Another possible function of exopolysaccharides is to act as signal molecules, thus causing the plant to perform a required step in nodule development. If this is the case, our results suggest that the disaccharide β-D-Glc*p*-(1→3)-D-Gal is part of the structure that is recognized by the plant.

EXPERIMENTAL

Culture of bacteria. — *R. meliloti* strain Rm8601 (Rm1021 expR101, exoA32::Tn5-233, Tn5) synthesizes EPS II as a consequence of the expR101 mutation, and fails to synthesize EPS I as a consequence of the exoA32::Tn5-233 mutation. This strain was grown in an M9-D-mannitol medium containing Na_2HPO_4, 22 g.L^{-1}; KH_2PO_4, 6 g.L^{-1}; NaCl, 2 g.L^{-1}; NH_4Cl, 2 g.L^{-1}; D-mannitol 1%, w/v; mM $MgSO_4$; 0.25mM $CaCl_2$; and 1 mg.mL^{-1} biotin. Ehrlenmeyer flasks containing M9-D-mannitol medium (250 mL) were inoculated with a stationary-phase Rm8601 culture (5 mL) and shaken for 3 days at 30°. The yield of EPS II was 270 mg.L^{-1} of culture supernatant. Strain Rm1021 produces 250 mg.L^{-1} of EPS II under nitrogen-starvation conditions[1].

Isolation of EPS II. — Cells were removed from cultures by centrifugation. The cell-free supernatant was dialyzed exhaustively against water, and lyophilized. The resulting crude exopolysaccharide was purified by chromatography on a Sephacryl S-500 (Pharmacia) gel-filtration column. Fractions containing exopolysaccharide were identified with the anthrone–H_2SO_4 test[16].

Preparation of oligosaccharide fragments of EPS II. — Oligosaccharide fragments of EPS II were obtained by treatment with a crude enzyme preparation derived from lysates of the *R. meliloti* transducing phage ϕM12. A lysate (100 mL) of ϕM12 grown on strain Rm7094 (Rm1021 exoB94::Tn5-233) was prepared as described by Finan *et al.*[17]. Cell debris was removed by centrifugation, and proteins in the supernatant were precipitated with 70% $(NH_4)_2SO_4$ and dissolved in depolymerase buffer (10mM HEPES, pH 7.4, 2.5mM $MgSO_4$, 2.5mM $CaCl_2$, and 50mM NaCl; 5 mL). This preparation was then dialyzed against depolymerase buffer at 4°. EPS II (10 mg) was dissolved in depolymerase buffer (1 mL), mixed with the enzyme preparation (1 mL), and incubated for 12–16 h at 30°. The resulting oligosaccharide fragments were applied to a Bio-Gel P-4 (BioRad, Rockville Center, NY) gel-filtration column, and eluted with water.

^{1}H-N.m.r. spectroscopy. — Samples for n.m.r. spectroscopy were lyophilized, treated several times with D_2O, and dissolved in D_2O at a concentration of 1–3 mg.mL^{-1}. Spectra were obtained with a 500-MHz instrument operated by the Francis National Magnet Laboratory at MIT, with the probe heated to 60°. The external chemical shift standard was sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

Mass-spectral analysis. — Fast atom bombardment-mass spectrometry (f.a.b.-m.s.) and desorption chemical ionization–mass spectrometry (d.c.i.–m.s.) of sugars were carried out on a VG ZAB-SE double-focusing mass spectrometer (VG Instruments, Beverly, MA) operating at 8 keV. For f.a.b.-m.s. analysis, and oligosaccharide sample was mixed with thioglycerol matrix (1 μL) before being loaded onto the stainless-steel target. The target was bombarded with Xe atoms with a kinetic energy of 8 keV. For d.c.–m.s. analysis, permethylated EPS II (~2 μg) was loaded onto the d.c.i. wire. Ammonia was used as the reagent gas.

Linkage analysis. — Partially methylated alditol acetates were prepared[9], and then analyzed with a Hewlett Packard 5890 g.l.c. and a Finnigan-MAT-312 g.l.c.–m.s. instrument (Finnigan-MAT, San Jose, CA). For g.l.c. separation, DB-5 capillary columns (30-m) were used which were temperature programmed by holding for 1 min at 75°, and then increasing to 150° at a rate of $20°.min^{-1}$, and finally to 300° at a rate of $2°.min^{-1}$. Identification of alditol acetates was achieved by comparison of g.l.c. relative-retention times and electron-ionization mass spectra with those of a library of standard compounds.

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