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CATION-DEPENDENT GELATION OF THE ACIDIC EXTRACELLULAR POLYSACCHARIDES OF *Rhizobium leguminosarum:* A NON-SPECIFIC MECHANISM FOR THE ATTACHMENT OF BACTERIA TO PLANT ROOTS

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ABSTRACT

The extracellular acidic heteropolysaccharides produced by various species of *Rhizobium leguminosarum* possess the same backbone but have different side-chains. Aqueous solutions of these polysaccharides form gels in the presence of excess of salt and divalent cations are more effective at inducing gelation than are monovalent cations, although the moduli of the gels are similar at comparable ionic strength. Gelation of the extracellular polysaccharide is proposed as a mechanism for attaching the bacteria to the tips of plant roots.

INTRODUCTION

Bacteria provide a source of potential new industrial thickening and gelling agents. As part of a search for useful polysaccharides, the rheological properties of the extracellular acidic polysaccharides (EPS) produced by certain *Rhizobium* species have been examined and we now report on the EPS produced by *R. leguminosarum* bv *viciae* strain 8401 pRL1JI. This strain is nearly isogenic with the wild type *R. leguminosarum* bv *phaseoli* strain 8002 and was derived from it by removal of the "phaseoli" symbiotic plasmid[1] pRP2JI and subsequent introduction of "viciae" symbiotic plasmid pRL1JI by conjugation[2]. Whereas strain 8002 infects *Phaseolus* (beans), the strain 8401 pRL1JI infects *Pisum* (peas).

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EXPERIMENTAL

R. leguminosarum strain 8401 pRL1JI was grown in Y medium, which is similar to the glutamic acid–mannitol–salts medium used by Zevenhuizen[3] but contains higher concentrations of salts. The medium contained (per L) mannitol (10 g), K_2HPO_4 (0.22 g), $CaCl_2 \cdot 6\ H_2O$ (0.22 g), glutamic acid (1.1 g), $Mg_2SO_4 \cdot 7\ H_2O$ (0.1 g), $FeCl_3 \cdot 6\ H_2O$ (0.02 g), biotin (750 μg), thiamine (750 μg), and pantothenic acid (750 μg). The pH of the medium was adjusted to 6.8. Bacteria were grown in 750 mL of medium contained in 2-L flasks, shaken at 150 r.p.m. at 29°. Cultures were harvested in the late phase of exponential growth (~72 h). The culture broth was centrifuged at $23 \times 10^3 g$ for 30 min; the supernatant solution was decanted, and filtered through two layers of glass-fibre paper (Whatman GF/B) and then sequentially through Millipore filters (1.2, 0.8, and 0.65 μm). The filtrate was concentrated (×3) by rotary evaporation and the EPS was precipitated with 4–5 vol. of industrial alcohol. The precipitate was collected and washed several times with industrial alcohol, and a solution in distilled water was freeze-dried to give crude EPS.

To an aqueous (0.2%) solution of crude EPS (500 mL) was added sodium sulphate to 10mM. EPS was precipitated (2 h, 37°) with 50 mL of aqueous 3% (w/w) cetyltrimethylammonium bromide[4] (CTAB). The precipitate was collected, washed with distilled water (3 × 650 mL), dissolved in aqueous 10% NaCl (400 mL), and precipitated with acetone, and a solution in aqueous 10% NaCl (400 mL) was dialysed against aqueous 1% NaCl and then distilled water, and freeze-dried to give purified EPS.

Rheological measurements were made using an INSTRON 3250. Gels were studied with a parallel plate assembly and liquids measured using a cone-and-plate arrangement.

RESULTS AND DISCUSSION

The results of sugar analysis[5], methylation analysis[6,7], and measurements of the uronic acid content[8] of the purified and crude EPS were consistent with the structure **1** (Fig. 1) first reported by Robertson *et al.*[9]. Sugar analysis and methylation analysis of the supernatant solution, obtained after precipitation of EPS from the crude extract with CTAB, revealed small quantities of (1→2)-Glc*p* attributable to traces of cyclosophorans[10].

Despite the similar overall carbohydrate composition of purified and crude EPS, the rheological properties were different. At >2%, aqueous solutions of crude EPS formed gels at room temperature, whereas those of purified EPS remained fluid. The rheological properties of crude EPS samples were examined in detail. Solutions of crude EPS in distilled water at 95° were poured into plastic moulds, covered, stored at 4° overnight, warmed to room temperature (25°), and examined using an Instron 3250. The rheological properties of a 3% crude EPS gel are shown in Fig. 2a. A possible explanation for the gelation was the presence of additional

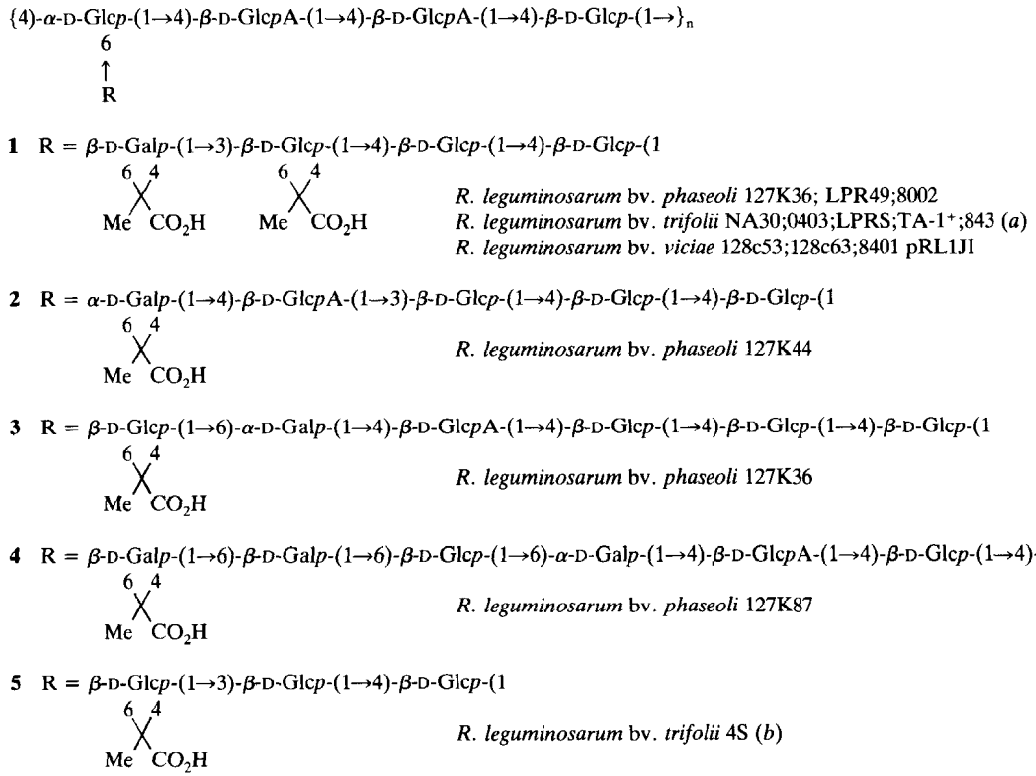

Fig. 1. Structures of the extracellular acidic heteropolysaccharides produced by *R. leguminosarum* species. The structures are as reported by McNeill *et al.*[11] except for (*a*) reported by Hollingsworth *et al.*[18] and Kuo and Mort[19] and (*b*) reported by Amemura *et al.*[20].

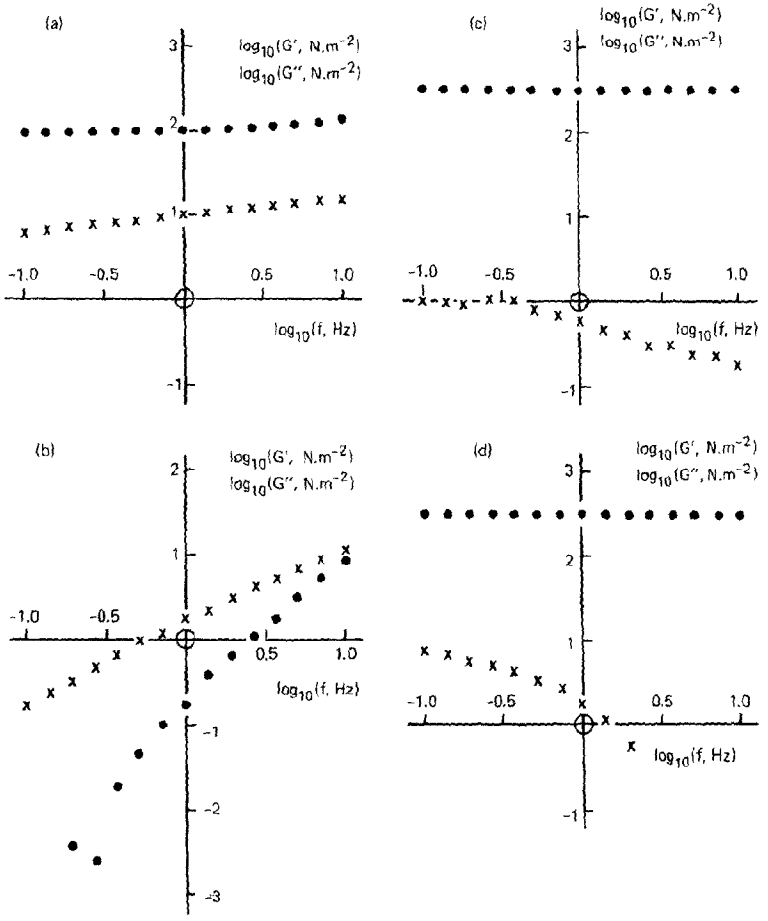

Fig. 2. Rheological data at 25° for samples of EPS from *R. leguminosarum* 8401 pRL1JI: (*a*) 3% gel of crude EPS, (*b*) 3% deionised aqueous solution of crude EPS, (*c*) 8% of deionised crude EPS gel containing 0.4M $CaCl_2$, (*d*) 3% of deionised crude EPS gel containing 1.6M NaCl. Data measured on an Instron 3250. Gels were studied using a flat-plate configuration and the liquids were studied using a cone-and-plate arrangement (cone angle, 2.4°): G′, storage modulus, •••; G″, loss modulus, x x x; f, frequency of oscillation.

salts in the medium. Therefore, an aqueous 0.2% solution of crude EPS was dialysed against distilled water, then passed over Dowex 50W-X8 (H^+) resin, neutralised with 0.1M NaOH, and freeze-dried. This procedure prevented gelation (Fig. 2b), but the addition of $CaCl_2$ resulted in the formation of thermoreversible gels (Fig. 2c). Addition of salts of other Group IIA divalent cations also led to gelation and the rheology of the gels was fairly independent of the type of cation (Table I). Addition of salts of monovalent (Group IA) cations at a similar ionic strength also caused gelation. Fig. 2d shows the properties of a gel obtained upon addition of NaCl.

TABLE I

RHEOLOGY[a] OF 3% GELS FORMED FROM DEIONISED CRUDE EPS OBTAINED FROM *R. leguminosarum* 8401 pRL1JI

Salt	*Molarity*	$G'(N.m^{-2})$	$G''(N.m^{-2})$	ϕ (°)
$MgCl_2$	0.4	459	2.80	3.5
$CaCl_2$	0.4	326	2.84	0.5
$SrCl_2$	0.4	341	6.55	1.1
$BaCl_2$	0.4	304	2.66	0.5
NaCl	1.6	314	1.64	0.3

[a]Data obtained using a flat-plate assembly on an Instron 3250. Values of G' (storage modulus), G'' (loss modulus), and ϕ (phase angle) were measured at a frequency of 1 Hz.

The structure of the EPS produced by *R. leguminosarum* 8401 pRL1JI is one of a family of structures (Fig. 1) produced by *R. leguminosarum* species. In order to examine whether gelation is likely to be a common feature of this family, the behaviour of the crude EPS produced by *R. leguminosarum* bv. *phaseoli* strain 127K87 (**4** in Fig. 1) was also examined. Members of this family differ in the length and composition of the side chain, and structures **1** and **4** represent the extremes. Gelation of structure **4** suggests that gelation is independent of the structure of the side chain. When grown in a supplemented salts medium (Y medium), this polysaccharide also formed gels, confirming the dependence of gelation on salt composition and concentration (Fig. 3). These studies demonstrate that the EPS of *R. leguminosarum* species will form gels in the presence of added salt and that divalent cations are most effective at inducing gelation.

R. leguminosarum species possess the ability to invade, cause nodule formation, and fix nitrogen in the roots of leguminous plants. The mechanisms of the

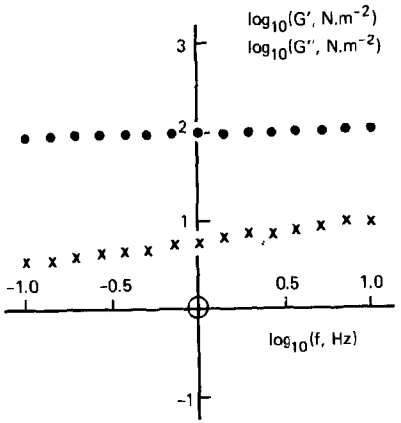

Fig. 3. Rheological data at 25° on a 3% gel of the crude EPS obtained from *R. leguminosarum* bv. *phaseoli* 127K87. Flat-plate configuration (Instron 3250). G' •••, G'' x x x.

symbiosis are largely unknown. Experimental evidence[12–14] suggests the involvement of EPS in the early stages of the interaction. Recent studies[9,11] suggest that EPS is unlikely to be involved in specific host recognition. However, gelation of the EPS provides a possible non-specific binding mechanism. A two-stage binding mechanism has been proposed[15]. Initial attachment of individual bacteria to plant roots involves[15] a calcium-dependent adhesion, although other Group IIA cations may also suffice. The second stage involves colony formation via the production of cellulosic fibrils[16], leading to the formation of caps. Gelation of the EPS of bacteria growing at the surface of the root tip, due to calcium (or other divalent cations) present in the soil, may aid the initial binding of individual *R. leguminosarum* bacteria. EPS^- mutants of *R. leguminosarum* strain 8401 pRL1JI do not nodulate or fix nitrogen in peas[17]. EPS^- mutants do cause root curling[17], suggesting that some degree of binding occurs. Even in the absence of the calcium-dependent adhesion step, limited cap formation may arise due to a specific adhesion of fibril-induced bacterial flocs[15].

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REFERENCES

1 J. W. Lamb, G. Hombrecher, and A. W. B. Johnston, *Mol. Gen. Genet.*, 186 (1982) 449–452.
2 J. A. Downie, G. Hombrecher, Q.-S. Ma, C. D. Knight, B. Wells, and A. W. B. Johnston, *Mol. Gen. Genet.*, 190 (1983) 359–365.
3 L. P. T. M. Zevenhuizen, *Appl. Microbiol. Biotechnol.*, 20 (1984) 393–399.
4 J. E. Scott, *Methods Carbohydr. Chem.*, 5 (1965) 38–44.
5 R. R. Selvendran, J. F. March, and S. G. Ring, *Anal. Biochem.*, 96 (1979) 282–292.
6 M. A. O'Neill and R. R. Selvendran, *Carbohydr. Res.*, 79 (1980) 115–124.
7 S. G. Ring and R. R. Selvendran, *Phytochemistry*, 17 (1978) 745–752.
8 N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.
9 B. K. Robertson, P. Aman, A. G. Darvill, M. McNeill, and P. Albersheim, *Plant Physiol.*, 67 (1981) 389–400.
10 L. P. T. M. Zevenhuizen, *FEMS Microbiol. Letts.*, 35 (1986) 43–47.
11 M. McNeil, J. Darvill, A. G. Darvill, P. Albersheim, R. van Veen, P. Hooykass, R. Schilperoort, and A. Dell, *Carbohydr. Res.*, 146 (1986) 307–326.
12 W. D. Bauer, T. V. Bhuvaneswari, A. J. Mort, and G. Turgeon, *Plant Physiol.*, 63 (1979) 5–135.
13 C. Napoli and P. Albersheim, *J. Bacteriol.*, 141 (1980) 1454–1456.
14 R. E. Sanders, R. W. Carlson, and P. Albersheim, *Nature (London)*, 271 (1978) 240–242.
15 G. Smit, J. W. Kline, and B. J. J. Lugtenberg, *J. Bacteriol.*, 169 (1987) 4294–4301.
16 M. H. Deinema and L. P. T. M. Zevenhuizen, *Arch. Microbiol.*, 78 (1971) 42–57.
17 D. Borthakur, Ph. Thesis, University of East Anglia, 1986.
18 R. I. Hollingsworth, F. B. Dazzo, K. Hallenga, and B. Musselman, *Carbohydr. Res.*, 172 (1988) 97–112.
19 M.-S. Kuo and A. J. Mort, *Carbohydr. Res.*, 145 (1986) 247–265.
20 A. Amemura, T. Harada, M. Abe, and S. Higashi, *Carbohydr. Res.*, 115 (1983) 165–174.