

# Nodule formation in soybeans by exopolysaccharide mutants of *Rhizobium fredii* USDA 191

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## Rhizobium fredii USDA191의 체외 다당류 결핍 변이주에 의한 근류 형성

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### 적 요

*Rhizobium*속 새균은 근체의 다당류를 생산하며, 이 다당류는 새균이 콩과식물 뿌리에 효과적으로 침입하여 뿌리혹을 형성하는 것과 관련이 있다고 알려져 왔다. transposon Tn5를 *Rhizobium fredii* USDA 191에 삽입시킴으로써 근체의 다당류 결핍변이주를 선발하였다. 그 중 표현형이 독특한 다섯개의 변이주를 선택하여, 그들의 체외다당류 합성능과 콩 뿌리혹 형성능에 대해서 조사하였다. 이들 변이주들에 의하여 생산된 근체의 다당류의 조성을 칼럼 크로마토그래피와 박층 크로마토그래피로 분석하였다. *exo-3*과 *exo-5* 변이주는 중성 glucan과 근체의 다당류를 생산하지 못했으나, *Glycine max* Peking에 접종하였을 때, 부분적으로 정상적인 뿌리혹을 유기생성시켰다. 나머지 3개의 변이주 *exo-1*, *exo-2* 그리고 *exo-4*는 각각 모균보다 많거나 적은 양의 중성 glucan을 생산하였고, 부분적으로 근체의 다당류를 결핍하고 있었다. 이런 결과로 볼 때, 근체의 다당류나 중성 glucan은 *R. fredii*가 뿌리혹을 형성하는데 반드시 필요한 것은 아니라고 추론할 수 있다.

### Introduction

The induction of nitrogen-fixing nodules on the roots of host legumes by *Rhizobium* and *Bradyrhizobium* bacteria is a multistep process involving interactions between the plant and its symbiont (see reviews by Halverson & Stacey, 1986; Keen & Staskawicz, 1988). Bacterial lipopolysaccharides and exopolysaccharides have been implicated as early bacterial signals in nodule development (Djordjevic *et al.*, 1987; Finan *et al.*, 1985; Gardiol *et al.*, 1987; Leigh *et al.*, 1985). Exopolysaccharides are composed of capsular polysaccharides (CPS) and extracellular polysaccharides (EPS) that differ chiefly in their degrees of polymerization (Tsien & Schmidt, 1981). Exopolysaccharide-deficient (*exo*) mutants of several *Rhizobium* species do not form normal nitrogen-fixing nodules; instead, they induce abnormal pseudonodules in legume roots which are devoid of bacteroids (Djordjevic *et al.*, 1987; Finan *et al.*, 1985; Noel *et al.*, 1986; Vandenbosch *et al.*, 1985). Several hypotheses for the functional role(s) of exopolysaccharides in the bacterial invasion process have been proposed. Exopolysacchar-

ides may function as signal molecules that induce host initiation of infection thread formation and allow the rhizobia to enter the plant (Downie & Johnston, 1986; Halverson & Stacey, 1986). They may function to allow the growth of the bacteria within the infection thread without evoking the plant defence mechanisms (Downie & Johnston, 1986; Verma & Nadler, 1984). They may act as host specificity factors which enable the bacteria to bind to specific plant lectins (Halverson & Stacey, 1986; Keen & Staskawicz, 1988). In short, exopolysaccharides may be functioning as direct inducers, indirect protectors, or both, in the bacterial penetration process.

The exopolysaccharides synthesized by various *Rhizobium* species can be divided into acidic polysaccharide and neutral polysaccharide components. Acidic exopolysaccharides are composed of hexoses and uronic acids (Huber *et al.*, 1984; Mort & Bauer, 1980, 1982; Robertsen *et al.*, 1981). The acidic exopolysaccharides of *R. fredii* USDA 191 contain uronic acid, mannose, galactose and glucose in an approximately 1:1:1:1 ratio and are also pyruvated (Lim & Tan, 1983). This is quite different from the reported composition of the exopoly-

saccharide from the more extensively studied species, *R. meliloti* 1021, which contains seven  $\beta$ -linked glucoses and one  $\beta$ -linked galactose and is modified by pyruvylation, acetylation and succinylation (Aman *et al.*, 1981). The neutral polysaccharide components contain primarily  $\beta$ -glucans (York *et al.*, 1980). Approximately 5 to 10% of the total polysaccharides secreted by fast-growing *Rhizobium* species are composed of  $\beta$ -2-linked glucans (York *et al.*, 1980). The  $\beta$ -glucans are cyclic compounds with about 20 glucose residues. There is a strong correlation between  $\beta$ -1,2-glucan synthesis and plant attachment in both *Rhizobium* infection and *Agrobacterium* virulence (Cangelosi *et al.*, 1987).

The present work was performed to determine if exopolysaccharide synthesis is a strict requirement for the induction of soybean nodulation by *R. fredii* USDA 191. Exopolysaccharide-deficient mutants of *R. fredii* USDA 191 were created by transposon Tn5 insertion mutagenesis, and their ability to induce nodulation in soybean roots was examined.

## Methods

**Biological materials.** Bacterial strains and plasmids are listed in Table I. *Escherichia coli* strains were maintained on Luria-Bertani (LB) medium (Maniatis *et al.*, 1982) and *R. fredii* on mannitol/salt/yeast extract (MSY) medium (Lim & Shanmugam, 1979). Antibiotics were added to the media at the following concentrations: 25  $\mu\text{g ml}^{-1}$  for kanamycin sulphate and chloramphenicol for *E. coli*; and 40  $\mu\text{g ml}^{-1}$  for nalidixic acid, 50  $\mu\text{g ml}^{-1}$  for kanamycin sulphate, 25  $\mu\text{g ml}^{-1}$  for streptomycin sulphate and 10  $\mu\text{g ml}^{-1}$  for tetracycline hydrochloride for *R. fredii*. Seeds of wild-type soybean, *Glycine soja* and *Glycine max* (Peking), obtained from R. Tully (USDA-ARS, Beltsville, Maryland, USA), were used for nodulation tests.

**Mutagenesis and screening for *exo* mutants.** Filter mating was done with a slight modification of the procedure of Hom *et al.* (1984). Cells from exponential-phase cultures of *E. coli* MM294A(pRK602) were mixed with cells of *R. fredii* YKL999, collected on a nitrocellulose membrane filter, washed three times with 0.9% NaCl, and incubated overnight on yeast extract/salt (YS) agar plates (Lim & Shanmugam, 1979) at 28 °C. Cells were washed off the filter with YS broth and dilutions were spread on MSY agar plates containing nalidixic acid, kanamycin sulphate and streptomycin sulphate (Selvaraj & Iyer, 1984). Small non-mucoid transconjugants were selected and purified by restreaking on the same selection medium.

EPS production was quantified from 7 ml of late-exponential-phase cultures grown in mannitol/glutamate (MG) medium (Sherwood, 1970) at 28 °C. The culture samples were centrifuged at 12000 *g* for 20 min to pellet the cells and EPS was precipitated from 5 ml of supernatant culture fluid by the addition of 2 vols acetone (Tully, 1985). This precipitate was washed three times with acetone/water (2:1, v/v), dried, and dissolved in 1% (v/v) H<sub>2</sub>SO<sub>4</sub>. Hexose content was determined by the anthrone method (Morris, 1948). The addition of acetone to the medium does not precipitate neutral glucans (Dudman & Jones, 1980).

**Plant nodulation test.** Method I. Seeds of *G. soja* were surface sterilized by soaking in concentrated H<sub>2</sub>SO<sub>4</sub> for 15 min, rinsing five times with sterile water for 5 min, washing with 50% (v/v) ethanol for

5 min, and rinsing again three times with sterile water for 2 min. Sterile seeds were germinated on 0.5% (w/v) water agar at room temperature in darkness for 3-4 d. Seedlings were inoculated by dipping the roots in a late-exponential-phase culture of bacteria for 30-60 s, and then transplanted to vermiculite containing nitrogen-free salt medium (Schwinghamer, 1960). Plants were harvested after 4 weeks of incubation in a growth chamber under fluorescent light with a 14 h photoperiod at 22 °C (night) and 30 °C (day). Every observable change in plant root tissue was noted. Four to five seedlings were inoculated with each bacterial strain and each experiment was performed twice.

**Method II.** Seeds of *G. max* (Peking) were surface-sterilized by successive soaking in 50% (v/v) ethanol (5 min) and 50% (w/v) sodium hypochlorite (5 min) and rinsing with copious amounts of sterile deionized water (Pueppke, 1983). Bacteria from late-exponential-phase cultures were collected by centrifugation, washed, and resuspended in water at a final concentration of 10<sup>9</sup> cells ml<sup>-1</sup>. Surface-sterilized seeds were soaked in this inoculum for 1 h and planted in vermiculite containing nitrogen-free salt medium (Schwinghamer, 1960) with 0.5 ml of the inoculum. The seeds were initially incubated in darkness for 4-5 d followed by the same regimen of temperature and light as above. Subsequent harvest and examination procedures were as described above.

**Recovery of bacteroids from nodules.** Nodules were excised from the roots and surface-sterilized in 50% (w/v) sodium hypochlorite for 2 min followed by five rinses with sterile water for 5 min. The nodules were then crushed in MSY medium and plated on selective tryptone/yeast extract (TY) agar plates (Jagadish & Szalay, 1984) to verify that the bacteroids which were recovered exhibited the same *exo* phenotype and antibiotic resistance as the original inoculum.

**Scanning electron microscopy.** Nodules were fixed with glutaraldehyde, dehydrated with ethanol, critical-point dried, sputter-coated (Postek *et al.*, 1980), and examined with an Hitachi S-500 scanning electron microscope.

**Fractionation and analysis of exopolysaccharide.** For size fractionation of EPS, *R. fredii* strains were grown in MG broth at 30 °C to an optical density at 600 nm of 0.6-0.8. Supernatant culture fluid was obtained after centrifugation at 6000 *g* for 30 min at 4 °C. The culture fluid was freeze-dried, dissolved in a minimal volume of water, and dialysed in 10 mM-phosphate buffer (pH 7.0) containing 0.02% sodium azide (elution buffer) at 4 °C in dialysis tubing with a molecular mass cut-off of 1000 Da.

For CPS isolation, bacteria were grown under the same conditions as above, bacterial cells were pelleted by centrifugation at 6000 *g* for 30 min, washed with phosphate-buffered saline (pH 7.2) (Mort & Bauer, 1980), and then resuspended in the same buffer for 6 d at 4 °C. The supernatant culture fluid containing the CPS was collected by centrifugation at 6000 *g* for 30 min at 4 °C, concentrated by freeze-drying and dialysed against deionized water.

EPS or CPS samples were size-fractionated by column chromatography. Bio-Gel A-5m (BioRad) columns (1.6 × 40 cm) were loaded with polysaccharide samples normalized for culture optical density and percentage of total crude supernatant culture fluid. The elution rate was approximately 0.07 ml min<sup>-1</sup> with elution buffer at 4 °C. Fractions of 1 ml were collected, and their hexose contents were determined by anthrone analysis.

EPS samples were further fractionated into acidic and neutral EPS by anion-exchange column chromatography. EPS samples were loaded on a DEAE-Sephadex A-50 (Pharmacia) column (6.5 ml) at 25 °C which was subsequently washed with deionized water until no hexose was detected in the effluent by the phenol/sulphuric acid method (Ashwell, 1966). Bound EPS was eluted from the column with 1 M-sodium acetate. For the identification of neutral glucans, those

fractions not binding to the anion exchanger were hydrolysed with 0.5 M-HCl at 100 °C for 5–20 min (Gardiol *et al.*, 1987). The products of acid hydrolysis were separated on silica gel thin-layer plates (Analtech), using a solvent mixture of 2-propanol/acetic acid/water (27:4:9, by vol.), and were visualized with orcinol ferric chloride reagent (Sigma) (Bonn *et al.*, 1986).

**Plasmid separation and DNA hybridization.** Plasmids were separated by agarose gel electrophoresis. Lysis of bacteria within the well (Hynes *et al.*, 1986) was used to separate the large plasmids of *R. fredii* by agarose gel electrophoresis. DNA was transferred to nitrocellulose filters by the method of Southern (Maniatis *et al.*, 1982) after partial depurination cleavage (Wahl *et al.*, 1979). A biotin-labelled Tn5 probe (Leary *et al.*, 1983) was prepared by nick-translating pSUP2021 DNA using a biotin-11-dUTP nucleotide (Bethesda Research Laboratories). DNA-DNA hybridization and detection of hybrids were done according to the manufacturer's procedure.

**Introduction of plasmids containing *R. meliloti* *exo* genes into *R. fredii* *exo* mutants.** Plasmids carrying functional *exo* genes from *R. meliloti* were obtained from G. Walker: pD56 (*exoF/B*), pD2 (*exoB*), pD5 (*exoD*), pD34 (*exoA*), pD15 (*exoC*) (Finan *et al.*, 1985). These plasmids, which are derivatives of the broad host-range plasmid pLAFRI, were transferred individually into the *R. fredii* *exo* mutants by triparental mating. The triparental matings were done using the helper plasmid pRK2013 by the filter mating procedure of Hom *et al.* (1984). Transconjugants were screened on MSY agar containing nalidixic acid, kanamycin sulphate and tetracycline hydrochloride.

## Results

### Isolation of *exo* mutants

Suicide plasmid pRK602 containing transposon Tn5 was mobilized from *E. coli* MM294A(pRK602) to *R. fredii* YKL999 by filter mating. From this mating, kanamycin-resistant transconjugants (Tn5 insertion mutants) of *R. fredii* were obtained at a frequency of  $1 \times 10^{-5}$  per recipient. *exo* mutants with a nonmucoid appearance on the selective medium were isolated at a frequency of about  $1 \times 10^{-3}$ . Eighty-five *exo* mutants were isolated and, of these, five phenotypically unique, nonmucoid colonies were chosen for further study (Table 1). Wild-type *R. fredii* colonies were highly mucoid in appearance and produced large, watery and opaque colonies. *R. fredii* YKL224 (*exo-1*) produced colonies that were nonmucoid and whiter in colour than the wild-type strain. YKL224 produced only 4% of the acetone-precipitable EPS of wild-type. YKL257 (*exo-2*) was slightly mucoid and produced 43% of the acetone-precipitable EPS of wild-type. YKL285 (*exo-3*) was translucent and produced 12% of the amount of EPS compared to the wild-type strain. *R. fredii* YKL288 (*exo-4*) and YKL293 (*exo-5*) were nonmucoid and produced only 2% and 4%, respectively, of acetone-precipitable EPS compared to the wild-type. In addition, YKL293 (*exo-5*) grew slowly and formed smaller colonies than the other *exo* mutants.

*R. fredii* USDA 191 contains at least three large

plasmids of 65 kb, 229 kb, and > 300 kb in size (Appelbaum *et al.*, 1985; Heron & Pueppke, 1984). DNA hybridizations probing for Tn5 insertions within these plasmids were performed on the five *exo* mutants. Bacteria were lysed within an agarose gel well, the three large plasmids were separated by agarose gel electro-

Table 1. *Bacterial strains and plasmids*

Strains and plasmids	Characteristics	References
<b>Strains</b>		
<i>Escherichia coli</i>		
SM10	C600, <i>recA</i> , Mu <sub>2</sub> * with integrated plasmid RP4-2-Tc::Mu, Km <sup>r</sup> : host of pSUP2021 and pSUP202	Simon <i>et al.</i> (1983)
MM294A	<i>pro-82 thi-1 endoA1 hsdR17 supE44</i> , host of pRK602	Leigh <i>et al.</i> (1987)
<i>Rhizobium fredii</i>		
USDA 191	Wild-type	Barbour <i>et al.</i> (1985)
YKL224	Tn5-induced <i>exo-1</i> mutant of YKL999, Km <sup>r</sup> Sm <sup>r</sup>	} This study
YKL257	As YKL224, but <i>exo-2</i>	
YKL285	As YKL224, but <i>exo-3</i>	
YKL288	As YKL224, but <i>exo-4</i>	
YKL293	As YKL224, but <i>exo-5</i>	
YKL999	Na <sup>r</sup> derivative of USDA 191	
<b>Plasmids</b>		
pRK602	pRK2013 Nm <sup>r</sup> ::Tn9 containing Tn5, Km <sup>r</sup> Cm <sup>r</sup>	Leigh <i>et al.</i> (1987)
pSUP202	Ap <sup>r</sup> Cm <sup>r</sup> Tc <sup>r</sup>	Simon <i>et al.</i> (1983)
pSUP2021	pSUP202 Tc <sup>r</sup> ::Tn5, Ap <sup>r</sup> Cm <sup>r</sup> Km <sup>r</sup>	Simon <i>et al.</i> (1983)
pRK2013	Helper, Km <sup>r</sup>	Ditta <i>et al.</i> (1980)

Km, kanamycin; Sm, streptomycin; Na, nalidixic acid; Nm, neomycin; Cm, chloramphenicol; Ap, ampicillin; Tc, tetracycline.

phoresis and DNA was transferred to nitrocellulose filters. A biotin-labelled probe of pSUP2021 containing Tn5 was hybridized to the DNA on the nitrocellulose filters. A positive hybridization signal was detected from *E. coli* MM294A(pRK602) plasmid DNA as well as pSUP2021 unlabelled DNA, but was not detected from plasmid DNAs from any of the *R. fredii* *exo* mutants or *R. fredii* YKL999. Positive hybridization signals were detected around the agarose gel well on the top of the agarose gel where large chromosomal DNA fragments migrate (data not shown) suggesting that the Tn5 insertions may be located in the chromosome.

### Analysis of the exopolysaccharides produced by the *exo* mutants

Acetone-precipitable EPS is a measure of bulk EPS. In

order to determine whether the variant levels observed were due to changes in specific polysaccharide fractions, EPS samples from the culture fluid were separated by size on a Bio-Gel A-5m column (Fig. 1). Culture fluid from wild-type *R. fredii*, YKL999, contained a set of heterogeneous polysaccharides. High-molecular-mass material eluted in fractions just after the void volume.

Low-molecular-mass material continued to be eluted up to an elution volume of 85 ml, with a major peak between 65 and 75 ml (Fig. 1a). In contrast, all the *exo* mutants possessed polysaccharide that was much less heterogeneous. EPS extracted from strain YKL224 (*exo-1*) (Fig. 1b) eluted as one sharp peak between volumes 65 and 75 ml. YKL257 (*exo-2*) (Fig. 1f) and YKL288 (*exo-4*) (Fig. 1e) synthesized polysaccharides that were similar to YKL224 polysaccharide but with the addition of two minor peaks of high-molecular-mass polysaccharide fractions. *exo* mutants YKL285 (*exo-3*) (Fig. 1d) and YKL293 (*exo-5*) (Fig. 1c), displayed little polysaccharide even within the major peak. This residual peak had a brownish colour instead of the normal greenish-blue colour observed in YKL999, YKL224, YKL257 and YKL288, which suggests that it is not likely to be a hexose fraction. YKL285 had a trace amount of high-molecular-mass polysaccharide eluting after the void volume.

Since EPS consists of both acidic and neutral components, the polysaccharides were further fractionated on DEAE-Sephadex columns to examine the relative contributions of both types. Less than 5% of the total EPS of the wild-type was neutral polysaccharide. In contrast, EPS of strain YKL224 consisted of 55% neutral and 45% acidic polysaccharide (Fig. 2a). EPS of YKL288 was primarily of neutral polysaccharide, which in turn constituted 70% of the total EPS (Fig. 2b).

Complete acid hydrolysis of neutral EPS yielded only a single component, corresponding to glucose on silica gel thin-layer plates. Partial hydrolysis, on the other hand, produced glucose polymers of varying length. Thus, the neutral EPS fraction is probably a glucan. In Table 2, the amount of neutral glucan produced by the *exo* mutants is presented for comparison with the wild-type.

Since no notable differences were discernible between YKL285 (*exo-3*) and YKL293 (*exo-5*) based on EPS analysis, the production of CPS was examined in these strains. CPS from the wild-type strain, when analysed on a Bio-Gel A-5m column, migrated as a single peak of low-molecular-mass polysaccharide (Fig. 3a). Both YKL285 (Fig. 3b) and YKL293 (Fig. 3c) mutants exhibited very little if any CPS.

#### *Complementation of R. fredii exo mutants EPS with plasmids containing R. meliloti exo genes*

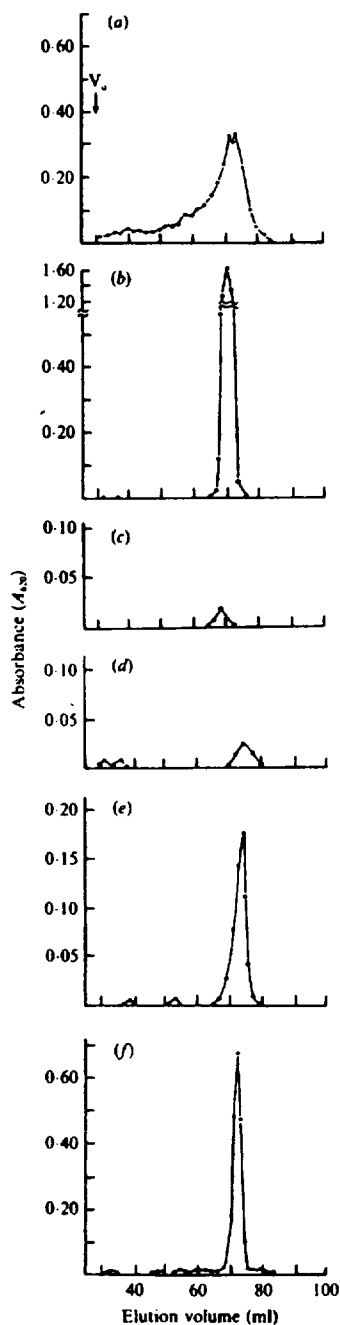


Fig. 1. Size-fractionation of EPS from supernatant culture fluid of wild-type and *exo* mutants. Culture samples were separated on Bio-Gel A-5m column, and the hexose content of the fractions was determined as described in Methods. The curves were normalized with respect to culture density and volume of culture fluid loaded.  $V_0$  void volume of the column. (a) YKL999 wild-type, (b) YKL224 (*exo-1*), (c) YKL293 (*exo-5*), (d) YKL285 (*exo-3*), (e) YKL288 (*exo-4*), (f) YKL257 (*exo-2*).

In *R. meliloti* 1021, six genetic loci involved in exopolysaccharide synthesis have been identified by gene cloning and complementation assays (Finan *et al.*,

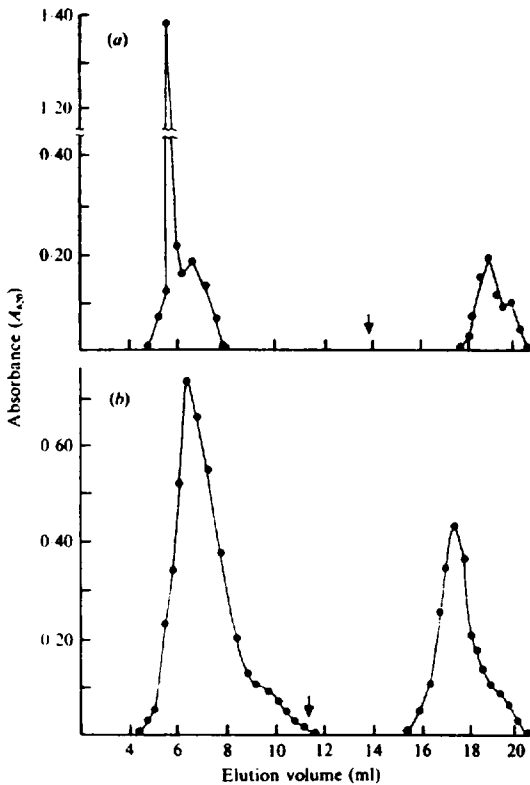


Fig. 2. Charge separation of neutral and acidic EPS. Equal volume of concentrated supernatant culture fluid from two *exo* mutants with similar optical density were separated on DEAE-Sephadex A-50 column. The column was eluted first with water, until no carbohydrate was detected in the effluent, and then with aqueous 1 M-sodium acetate. The arrow indicates the point of addition of 1 M-sodium acetate. (a) YKL224 (*exo-1*), (b) YKL288 (*exo-4*).

1985; Leigh *et al.*, 1985). Four of the *exo* mutations were mapped to megaplasmids within *R. meliloti*, and two mutations were located on the chromosome (Finan *et al.*, 1985; Leigh & Lee, 1988). Plasmids carrying functional *exo* genes from *R. meliloti* were obtained: pD56 (*exoF/B*), pD2 (*exoB*), pD5 (*exoD*), pD34 (*exoA*), pD15 (*exoC*) (Finan *et al.*, 1985). They were introduced individually into the *exo* mutants of *R. fredii* using triparental matings. The restoration of exopolysaccharide production was assayed. Transconjugants of YKL288 by pD56 (*exoF/B*), but not by pD2 (*exoB*), produced only 66% as much EPS as the wild-type, YKL999. Transconjugants of YKL293 by pD15 (*exoC*) produced 86% as much EPS as the wild-type. YKL285, YKL257 and YKL224 were not affected in EPS synthesis by the introduction of any plasmid encoding an *exo* gene from *R. meliloti*.

#### Nodulation ability of the *exo* mutants

The five *R. fredii* *exo* mutants were examined for ability to induce nodulation of soybean plants. The nodules that formed on the plant roots consisted of two types. The first class were typical spherical nodules that were large, determinate, and pink-pigmented. Bacteroids were recovered from these nodules after surface sterilization of the nodule. The second class of nodules were small, irregular in shape, and white, with a vascular bundle in the centre. No bacteroids could be recovered from these atypical nodules. Scanning electron micrographs of both nodule types are shown in Fig. 4. No bacteroids could be seen in cross-sections of the atypical 'pseudonodules' (Fig. 4e, f), whereas clusters of bacteroids were found inside the cortical layer of typical nodules (Fig. 4c, d).

The average numbers of nodules induced per plant by the wild-type and *exo* mutants of *R. fredii* USDA 191 on *G. soja* and on *G. max* are presented in Table 2.

Table 2. Comparison of the nodulation ability and exopolysaccharide production of *R. fredii* USDA 191 wild-type and *exo* mutants

Strains	Number of nodules per plant (T/A)*		Neutral glucan fraction† (% of wild-type)	CPS‡ (% of wild-type)	EPS§ (% of wild-type)
	<i>G. soja</i>	<i>G. max</i>			
YKL999 (wild-type)	1.0 <sup>a</sup> /10	3.4 <sup>c</sup> /0.0	100.0	100.0	100
YKL224 ( <i>exo-1</i> )	1.2 <sup>a</sup> /20	0.1 <sup>f</sup> /2.1	235.0	ND	4
YKL257 ( <i>exo-2</i> )	1.0 <sup>a</sup> /32	0.1 <sup>f</sup> /4.7	100.0	ND	43
YKL285 ( <i>exo-3</i> )	1.3 <sup>a</sup> /26	1.3 <sup>ab</sup> /15.2	2.5	4.0	12
YKL288 ( <i>exo-4</i> )	0.4 <sup>ab</sup> /15	2.0 <sup>a</sup> /3.4	25.0	ND	2
YKL293 ( <i>exo-5</i> )	0.0 <sup>b</sup> /16	0.5 <sup>e</sup> /9.4	1.0	4.2	4
Control	0.0 <sup>b</sup> /0.0	0.0 <sup>f</sup> /0.0	NA	NA	NA

ND, not determined. NA, not applicable.

\* Four to five seedlings of soybean were inoculated with each strain for two separate experiments and repeated once. The numbers are LS (least squares) means of typical (T) nodules and average numbers of atypical (A) nodules per plant. LS means superscripted by the same letter are not significantly different ( $P = 0.05$ ) according to least significant difference tests performed on individual LS means.

† Neutral EPS fraction was determined from the elution profile on a DEAE-Sephadex A-50 column.

‡ CPS was estimated from the elution profiles on a Bio-gel A-5m column (Fig. 3).

§ EPS was determined by addition of 2 vols acetone to culture supernatant.

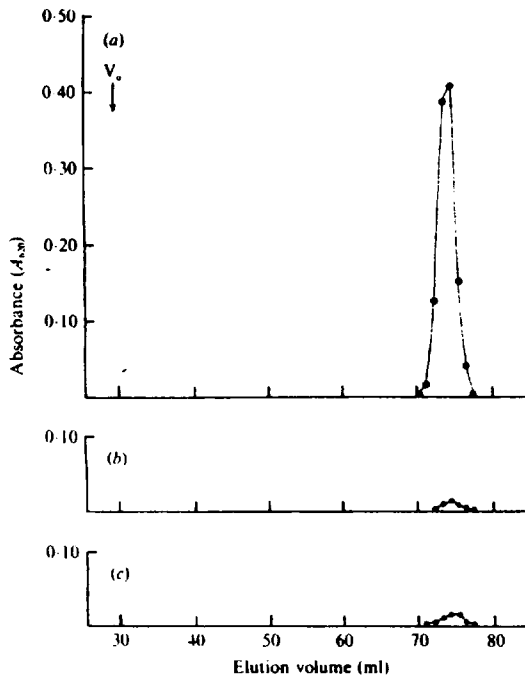


Fig. 3. Size fractionation of CPS from wild-type and two *exo* mutants of *R. fredii* USDA 191. CPS isolated as described in Methods were separated on a Bio-Gel A-5m column. The curves were normalized with respect to culture density and volume of culture fluid loaded.  $V_0$ , void volume of the column. (a) YKL999 (wild-type). (b) YKL285 (*exo-3*), (c) YKL293 (*exo-5*).

YKL293 (*exo-5*) was the only mutant strain that did not induce any typical spherical nodules on *G. soja*. The other four mutant strains induced typical nodules comparable in number to those induced by the wild-type strain YKL999. Bacteria recovered from the typical nodules induced by the *exo* mutants were phenotypically the same with regard to EPS production and antibiotic resistance as the inoculating bacteria. The low number of spherical nodules observed per plant (approximately one per root) was typical for wild-type *R. fredii* infection of this soybean (Heron & Pueppke, 1984). Interestingly, *exo-5* mutant YKL293, as well as other *exo* strains and the wild-type, induced the formation of many small atypical 'pseudonodules' on the infected roots of *G. soja*.

*R. fredii* was originally isolated from *G. max* (Peking) and this plant appears to be a preferred host (Keyser *et al.*, 1982). When these same *exo* mutants were tested for nodulation on *G. max*, the nodulation efficiencies of mutants were different from those obtained on *G. soja*. Soybeans inoculated with YKL224 (*exo-1*) and YKL257 (*exo-2*) developed spherical nodules in only one plant in ten, though all plants produced two or more atypical pseudonodules. In contrast to *G. soja*, *G. max* inoculated

with YKL293 (*exo-5*) developed typical nodules 50% of the time, and when inoculated with YKL288 (*exo-4*) it nodulated only slightly less than when inoculated with wild-type. A comparison of the nodule induction by *R. fredii* *exo* mutants and EPS production (last column of Table 2) indicated no strong correlation between EPS levels and ability to induce nodulation.

## Discussion

The major question addressed in this paper is whether *exo* mutants of *R. fredii* USDA 191 are adversely affected in the induction of soybean nodulation. In *R. meliloti*-alfalfa symbiosis, bacterial exopolysaccharide synthesis was found to be essential in the induction of nodule formation: all the *exo* mutants of *R. meliloti* 1021 except *exoD* showed lack of nodule invasion and induced only pseudonodules in alfalfa roots (Leigh *et al.*, 1985, 1987; Leigh & Lee, 1988). Similarly, *exo* mutants of *R. leguminosarum* were found not to induce effective nodule formation on peas (Borthakur *et al.*, 1986). Both *R. meliloti* and *R. leguminosarum* form cylindrical indeterminate nodules on the roots of their respective hosts. In fact, the requirement for sufficient EPS appears to be stringent only for indeterminate nodule formation.

When Chen *et al.* (1985) tested Tn5-induced mutants of *Rhizobium* NGR234 (a strain with a broad host range) for nodulation on determinate and indeterminate nodule-forming hosts, these *exo* mutants induced normal-to-small nodules on the determinate host's roots, but induced only small callus-like structures on the indeterminate host's roots. Similarly, Borthakur *et al.* (1986), working with the same gene found that a single *exo* mutation in *R. leguminosarum* made the bacterium ineffective in the nodulation of peas, but in *R. phaseoli* had no effect on nodulation in beans. Thus, the dependence on exopolysaccharide synthesis for nodulation of plants appears to be stronger for the indeterminate nodule structure.

Our data (Table 2) with *exo* mutants of *R. fredii* USDA 191 are consistent with the above correlation. *R. fredii* USDA 191 induces determinate nodules on soybeans. There was no apparent absolute requirement of exopolysaccharides for nodulation of *G. max* or *G. soja*. Four *exo* mutants of *R. fredii*, YKL224 (*exo-1*), YKL257 (*exo-2*), YKL285 (*exo-3*) and YKL288 (*exo-4*) induced typical spherical nodules on *G. soja*. Only one mutant, YKL293 (*exo-5*), was found unable to induce nodulation in this plant species. However, the *exo-5* mutation must be considered pleiotropic because the mutant cells grew slowly compared with other *exo* mutants and the wild-type. The infection of *G. soja* with *exo* mutants and wild-type induced the production of a large number of small pseudonodules. This induction of

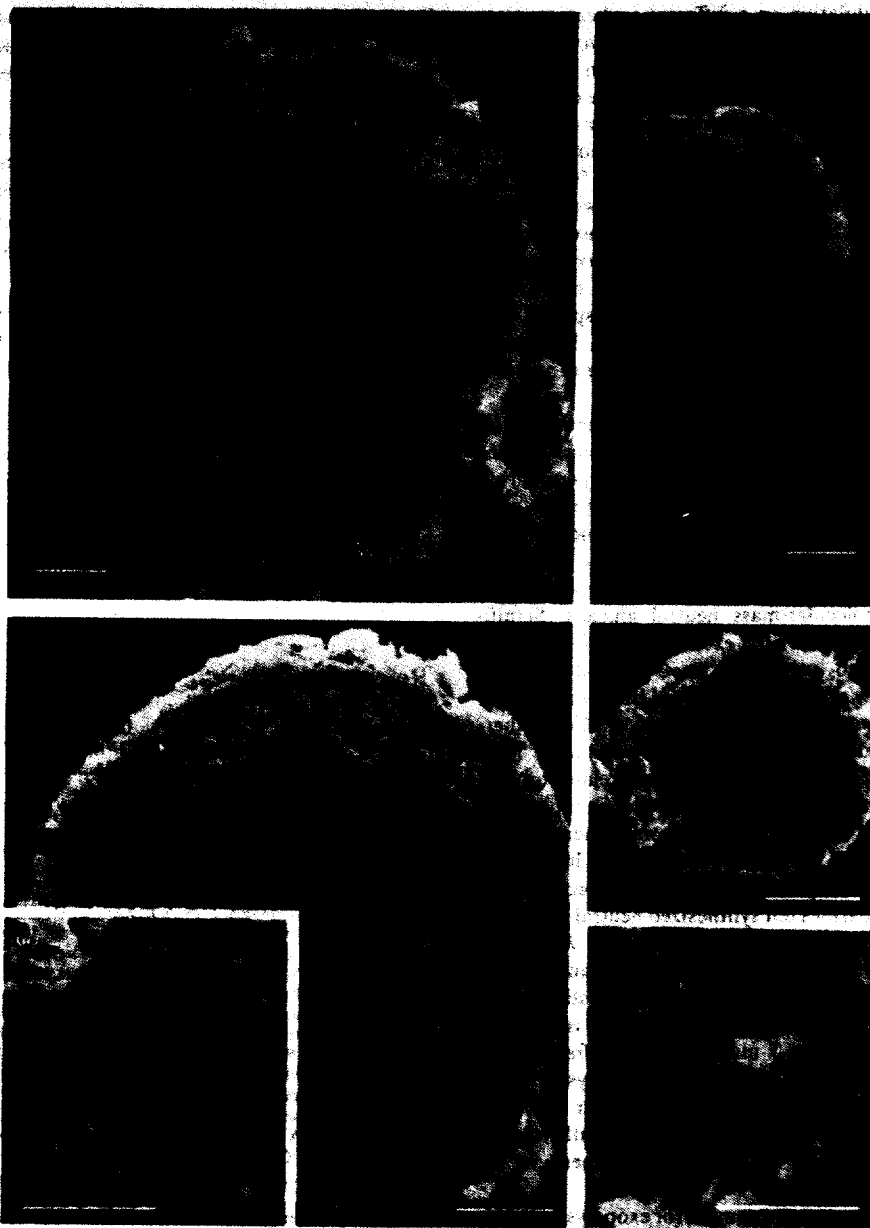


Fig. 4. Scanning electron micrographs of nodules induced in *G. soja* roots by *R. fredii* USDA 191. (a, b) Typical nodule and atypical nodule induced in *G. soja* roots by *R. fredii* USDA 191 after 4 weeks growth. (c) Cross-section of typical nodule and (d) enlargement of typical nodule centre. (e) Cross-section of atypical nodule and (f) enlargement of atypical nodule centre. Bar, 500  $\mu\text{m}$  (a, b, c and e), bar, 5  $\mu\text{m}$  (d and f).

plant cell differentiation without concomitant bacterial invasion indicates that it is not a preferred plant host of *R. fredii* USDA 191.

We tested nodulation on a different soybean species, *G. max* (Peking). Correlating with *G. max* being a preferred host for *R. fredii*, there was an increased number of nodules per plant root, an absence of atypical nodules upon wild-type infection and a lower number of atypical nodules from *exo* mutant infections. YKL-293 (*exo-5*) was able to induce nodules on 50% of the infected roots of this soybean. However, two different *exo* mutants, YKL224 (*exo-1*) and YKL257 (*exo-2*), were very inefficient in nodule formation in *G. max*, though a normal nodule could be found in one of ten plant roots infected. Interestingly, two mutants that displayed low production of neutral glucan fractions, YKL285 (*exo-3*) and YKL293 (*exo-5*), induced a large number of atypical nodules in *G. max*.

We tested for exopolysaccharide complementation by *R. meliloti* *exo* gene clones because *Rhizobium* species may share common pathways in exopolysaccharide synthesis. YKL293 (*exo-5*) was complemented by a *R. meliloti* *exoC* gene clone. This was a pleiotropic mutation that affected strain growth rate and resulted in loss of both the high-molecular-mass acidic exopolysaccharides and the low-molecular-mass neutral glucans. Significantly, the phenotype of the *exoC* mutant of *R. meliloti* 1021 is similar to *exo-5*. The *exoC* mutant was also pleiotropic, grew slowly and produced little if any acidic or neutral polysaccharide (Leigh & Lee, 1988). Furthermore, recent studies of *Agrobacterium tumefaciens* indicate that it has a gene, *pscA*, that is closely related to *exoC* and a *pscA* mutant was defective in  $\beta$ -glucan production (Kamoan *et al.*, 1989). YKL288 was complemented by *R. meliloti* *exoF/B* but not by an *exoB* clone. This mutant was defective in the synthesis of the high-molecular-mass acidic polysaccharides but synthesized neutral glucans at about 25% of normal. The *exoF* mutant *R. meliloti* appears to have a similar phenotype because it was defective in succinoglycan production but still synthesized  $\beta$ -glucans (Leigh & Lee, 1988). Thus it is probable that the *exo-3* and *exo-5* mutations of *R. fredii* are in genes from a common pathway in exopolysaccharide synthesis. However, these probably similar *exo* gene mutations affect induction of nodules differently when in *R. fredii* USDA 191 compared to when in *R. meliloti* 1021.

We conclude that a requirement for exopolysaccharide production is not as critical in the formation of determinate nodules as it is for formation of indeterminate nodules. This probably relates to the distinctly different mechanisms of morphogenesis observed for the two forms of nodules (Goodchild, 1977; Newcomb, 1981).

## SUMMARY

Production of exopolysaccharides by *Rhizobium* has been linked with efficient invasion and nodulation of leguminous plant roots by the bacteria. Exopolysaccharide-deficient (*exo*) mutants of *Rhizobium fredii* USDA 191 were isolated following Tn5-insertion mutagenesis. Five phenotypically unique *exo* mutants were investigated for exopolysaccharide synthesis and their ability to nodulate soybeans. The exopolysaccharides produced by these mutants were analysed for polysaccharide composition by column chromatography and thin-layer chromatography. Two mutants designated *exo-3* and *exo-5* were deficient in both neutral glucan and exopolysaccharide synthesis, but each induced some functional nodules on *Glycine max* (Peking). The remaining three mutants (*exo-1*, *exo-2* and *exo-4*) synthesized neutral glucans at levels higher or lower than those in the wild-type and exhibited partial exopolysaccharide deficiencies. The data imply that neither exopolysaccharides nor neutral glucans are essential for the induction of determinate nodules by *R. fredii*.

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