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Nitrogen Fixation Ability of Exopolysaccharide Synthesis Mutants of *Rhizobium* sp. Strain NGR234 and *Rhizobium trifolii* Is Restored by the Addition of Homologous Exopolysaccharides

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Several transposon Tn*5*-induced mutants of the broad-host-range *Rhizobium* sp. strain NGR234 produce little or no detectable acidic exopolysaccharide (EPS) and are unable to induce nitrogen-fixing nodules on *Leucaena leucocephala* var. Peru or siratro plants. The ability of these Exo$^-$ mutants to induce functioning nodules on *Leucaena* plants was restored by coinoculation with a Sym plasmid-cured (Nod$^-$ Exo$^+$) derivative of parent strain NGR234, purified EPS from the parent strain, or the oligosaccharide from the EPS. Coinoculation with EPS or related oligosaccharide also resulted in formation of nitrogen-fixing nodules on siratro plants. In addition, an Exo$^-$ mutant (ANU437) of *Rhizobium trifolii* ANU794 was able to form nitrogen-fixing nodules on white clover in the presence of added EPS or related oligosaccharide from *R. trifolii* ANU843. These results demonstrate that the absence of *Rhizobium* EPSs can result in failure of effective symbiosis with both temperate and subtropical legumes.

A complex multistep interaction between the soil bacterium *Rhizobium* and specific leguminous plants results in the induction of nitrogen-fixing nodules on legume roots (19 and references therein). The early steps of the interaction are characterized by the distortion or curling of the root hair cells. The cell walls of the root hairs are penetrated after 24 h by a compatible *Rhizobium* strain, and an infection thread is synthesized by the plant after the nucleus of this cell has migrated to the infection site (4, 17). The bacteria are carried toward the root cortex inside the infection thread, where they actively divide. Shortly before or concurrent with initiation of infection thread synthesis, cortical cell division is thought to be induced by the *Rhizobium* strain, presumably by diffusible substances released by the bacterium (1, 2).

Another feature of the *Rhizobium*-legume interaction is the host specificity displayed. Fast-growing ("temperate") *Rhizobium* strains, for example, usually nodulate only one plant species effectively, whereas slow-growing *Bradyrhizobium* strains typically have a broad host range. In contrast, the fast-growing *Rhizobium* sp. strain NGR234 (28) possesses an unusually extensive host range, which includes a variety of tropical and temperature legumes as well as the nonlegume tropical tree *Parasponia andersonii* (29).

Since the initial interaction between the symbionts occurs at the surface of the two organisms, cell surface molecules may be important in determining the outcome of the infection. Rhizobia characteristically produce large amounts of exopolysaccharides (EPSs) on various laboratory media, and the colonies formed are mucoid (Muc$^+$) in appearance. Cell mixing experiments involving spontaneous mutant derivatives of *Rhizobium trifolii* NA34 indicated that EPS may be important for nodule development (22). Two different spontaneous mutants of strain NA34 were identified: an invasive Muc$^-$ strain, SU846, and a Muc$^+$ Nod$^-$ strain, SU847. The Muc$^+$ Nod$^-$ strain SU847 has an extensive deletion in the native Sym plasmid which removes many essential nodulation and nitrogen fixation genes (M. A. Djordjevic, Ph.D. thesis, Australian National University, Canberra City, 1983). The Muc$^-$ Nod$^+$ strain inoculated alone onto clover plants formed poorly developed nodules which were unable to fix nitrogen. However, a mixed inoculum containing these two strains could induce some fully functional nitrogen-fixing nodules on clovers (22).

Rhizobium polysaccharides, particularly EPSs and lipopolysaccharides, have been postulated to be involved in the infection and nodulation of legumes (24, 25), including specific adhesion to the root hair surfaces (9) and the determination of host specificity (13). Exo$^-$ mutants of *Rhizobium meliloti* are apparently affected at an early stage of infection (15). In other species, EPS may play a role at later stages of infection. A number of transposon Tn*5*-induced Exo$^-$ mutants of *R. trifolii* (6) and strain NGR234 (7) are still able to infect host legumes but are unable to initiate nitrogen-fixing nodules. Examination of nodules induced by Exo$^-$ mutants of *R. trifolii* and strain NGR234 by electron microscopy has shown that nodules on some plant species are poorly developed, contain fewer dividing cells and intracellular bacteria, and give little or no indication of the presence of bacteroids (6, 7, 23).

The approach we have used to determine the role of EPS in symbiosis involved the isolation of specific nonmucoid mutants. This involved the isolation of more than 90 Tn*5*-induced EPS-defective strains of NGR234; these mutations were mapped on the chromosome of this strain (7). We have been able to determine the precise structure of the EPS of the broad-host-range strain NGR234 (11, 12). In this paper we show that several Tn*5*-induced Exo$^-$ mutants of NGR234 which produce very little or no acidic-type EPS fail to induce nitrogen-fixing nodules on several host plants. In addition, these Exo$^-$ mutants can have their symbiotic phenotypes corrected by the addition of either purified EPS from NGR234 or the oligosaccharide repeat unit from which the polymer is built. This result demonstrates that *Rhizobium*

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TABLE 1. Bacterial strains

Strain	Reference	Relevant genetic characteristics	Location of Tn*5* in *Eco*RI-digested total DNA (kb)	Phenotype of plant response		White clover
				Leucaena	Siratro	
NGR234 derivatives						
ANU280	7	Parent strain, Sm^r Rf^r derivative of NGR234 (Exo$^+$)	Tn*5* not present	Large Nod$^+$ Fix$^+$ nodules	Large Nod$^+$ Fix$^+$ nodules	
ANU265	16	Sym plasmid-cured derivative of NGR234; Sm^r Sp^r (Exo$^+$)	Tn*5* not present	Nod$^-$	Nod$^-$	
ANU2811	7	Tn*5*-induced Exo$^-$ mutant of ANU280; Km^r Sm^r Rf^r	7.0	Callus (Fix$^-$)	Nod$^-$	
ANU2840	7	Tn*5*-induced Exo$^-$ mutant of ANU280; Km^r Sm^r Rf^r	7.5	Callus (Fix$^-$)	Large Nod$^+$ Fix$^+$ nodules	
ANU2820	7	Tn*5*-induced Exo$^-$ mutant of ANU280; Sm^r Rf^r Km^r	18.0	Callus (Fix$^-$)	Small Fix$^-$ nodules	
R. trifolii						
ANU794	6	Exo$^+$ Sm^r	Tn*5* not present			Nod$^+$ Fix$^+$ nodules
ANU437	6	Tn*5*-induced Exo$^-$ derivative of ANU794; Sm^r Km^r	9.4			Nod$^+$ Fix$^-$ nodules
ANU843	26	Parent strain; Exo$^+$	Tn*5* not present			Nod$^+$ Fix$^+$ nodules
ANU845	26	Sym plasmid-cured derivative of ANU843, Exo$^+$	Tn*5* not present			Nod$^-$

EPSs play an essential role in the development of nitrogen-fixing nodules.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1.

Media. All media used (BMM, Fahraeus) have been described earlier (21).

Coinoculation experiments. Exo$^-$ mutant strains of ANU280 and Sym plasmid-cured derivatives of strains ANU280 and ANU843 were grown on BMM agar plates for 2 days. Suspensions of both strains were grown to the same optical density and mixed in an appropriate 1:1 ratio before inoculation onto sterile 3-day-old *Leucaena leucocephala* var. Peru seedlings.

Isolation and purification of polysaccharides. Strains ANU280, ANU2811, ANU2820, and ANU2840 were grown in a glutamic acid–D-mannitol–salts medium as described earlier (12). Cells and extracellular polysaccharide were separated from material of lower molecular weight with an Amicon DC10L hollow fiber filtration system fitted with a 0.1-μm cutoff filter. The cells were then separated from the polysaccharide by centrifugation at 7,000 × *g* for 15 min. The filtrate was passed through a second hollow fiber filter (H10P3-20) which retained oligosaccharides of molecular weight greater than 1,500. Small molecules were removed by exhaustive diafiltration with distilled water. The retentates were freeze-dried.

EPS was further purified as the cetyltrimethylammonium salt as described previously (12). The oligosaccharide fraction was separated on a DEAE-Sephadex A-25 column with a salt gradient as described earlier (12) and analyzed for hexose and uronic acid. The fractions thus isolated were rechromatographed over Biogel P2 at pH 3.3 (11). The identity of the oligosaccharide was established by carbon-13 nuclear magnetic resonance (NMR) spectroscopy. All the observed resonances were consistent with a structure corresponding to the repeat unit described previously (12), with additional decoration by acetate at sites that have not been determined. The chemical shifts for the C1 carbons at the reducing terminus of the molecule were 93.4 and 97.4 ppm, demonstrating that the terminal sugar is galactose, not glucose. The intensities of the C1 carbons of the various sugars were compared by integrating spectra obtained with gated proton decoupling to suppress nuclear Overhauser enhancement. These intensities indicated that the oligosaccharide was a nonasaccharide with two α-glucuronosyl residues, a nonreducing α-galactose, and five β-linked pyranose rings assigned to glucose, in addition to the reducing terminal galactose.

Other unambiguous assignments can be made from chemical shift information reported previously (12): the nonreducing galactose carries a 4,6-pyruvylidene substituent; on average, one pyruvate and less than one acetate group. The reducing terminal galactose was absent from about 10% of the molecules.

There was therefore a degree of heterogeneity in the sample. Most of the material has the structure shown in Fig. 2A, but small amounts may have related structures such as those lacking galactose or acetate.

Sterile seedlings germinated on BMM agar were transferred to Fahraeus agar and allowed to settle to the agar surface for 16 h. Portions (5 or 10 μl) of stock solutions (5 mg/ml) of EPS or its derived oligosaccharide were applied along the length of 3-day-old seedlings. These acidic saccharides were added either simultaneously or 24 h before or after inoculation with rhizobia.

Plant assays. *Macroptilium atropurpureum* (DC) Urban (siratro), which produces determinate nodules, and *Trifo-*

lium repens (L) cv 5826 (white clover), an indeterminate nodule former, were tested for nodulation by the plate method as described previously (5, 21). *L. leucocephala* (Lam) Wit. var. Peru, which forms an indeterminate nodule, was tested for nodulation by the method described previously (7).

Preparation of nodule sections for light microscopy. Specimens for light microscopy were prepared as described earlier (7).

RESULTS

EPS mutants. Mutants ANU2811, ANU2820, and ANU2840 are Tn*5*-induced mutants of strain NGR234 defective in EPS synthesis. These strains form nonmucoid (Muc$^-$) colonies on all laboratory media and have defective symbiotic phenotypes on different legume plants (7). The Tn*5* insertion in these strains has been shown to reside in different genomic DNA fragments (Table 1). Little or no uronic acid-containing saccharides could be detected by colorimetric analysis of both retentates obtained by hollow-fiber filtration. Chromatographic analysis of the oligosaccharide fraction from these Exo$^-$ strains showed that they failed to produce any detectable acidic oligosaccharide (Fig. 1). The large peak in Fig. 1A (X) contained the acidic oligosaccharide repeat unit (Fig. 2). Note the absence of this peak in the elution profile of strain ANU2840. The elution profiles of Exo$^-$ strains ANU2811 and ANU2820 also lacked any trace of the oligosaccharide repeat unit. Peaks 1 to 5 represent the elution positions of glucans substituted with glycerol-phosphate residues. In contrast, the parent strain ANU280 produced gram quantities of acidic EPS and related oligosaccharide when grown under the same culture conditions. Although Exo$^-$ strain ANU2840 failed to produce any detectable acidic oligosaccharide (Fig. 1), colorimetric analysis of the retentate from strain ANU2840 indicated that a small amount of acidic EPS may have been produced. In view of the total absence of acidic oligosaccharide, this apparent low level of uronic acid-containing material in the retentate may be an artifact. Despite the different location of Tn*5* in each of these Exo$^-$ mutants, strains ANU2811, ANU2840, and ANU2820 had a common nodulation-defective phenotype on *Leucaena* plants but a different phenotype on siratro plants. On the roots of *Leucaena* plants these Exo$^-$ mutants produced grossly disorganized calluslike structures which did not fix atmospheric nitrogen, whereas the parent strain induced indeterminate nitrogen-fixing nodules (7). On siratro, the Exo$^-$ mutants ANU2820 and ANU2811 produced poor, non-nitrogen-fixing nodules, while strain ANU2840 forms nitrogen-fixing nodules on a proportion of plants (7).

Coinoculation of Exo$^+$ and Exo$^-$ strains on *Leucaena* plants. The Sym plasmid-cured derivative of NGR234, ANU265, produces mucoid colonies, but is unable to initiate any detectable symbiotic response on any legume since all

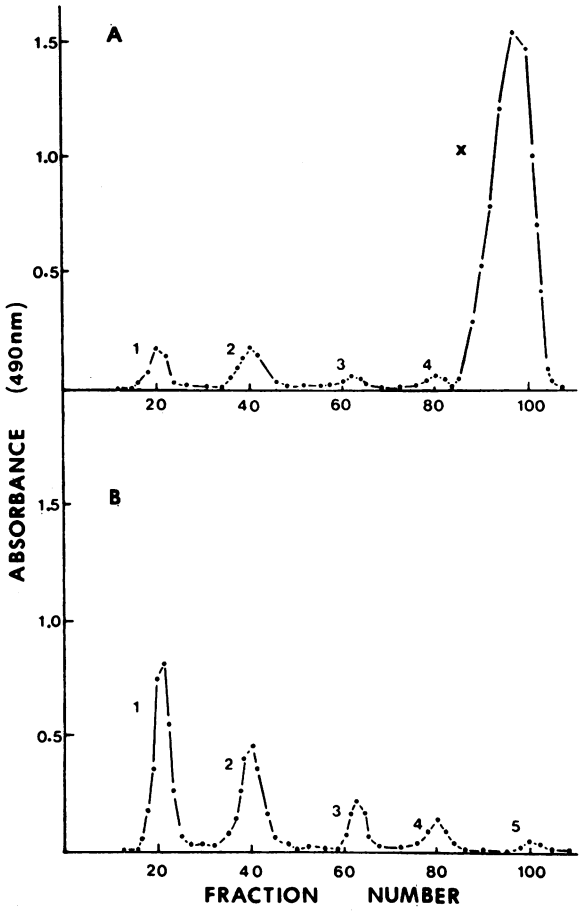

FIG. 1. Chromotographic analysis of the oligosaccharides secreted from *Rhizobium* sp. strain NGR234. The oligosaccharide fraction (H10P3-20 retentate) was eluted from a column (1.5 by 40 cm) of DEAE-Sephadex A-25 equilibrated with 25 mM Tris hydrochloride (pH 7.5). Neutral components were eluted at 8 ml/h with 90 ml of the same buffer, and acidic components with a convex gradient formed from 90 ml of 25 mM Tris hydrochloride and 100 mM sodium chloride in 25 mM Tris hydrochloride (limit buffer, 300 ml). Portions of the 2.0-ml fractions were analyzed for hexose and uronic acid. (A) Elution profile of oligosaccharides from the parent strain ANU280. (B) Elution profile of oligosaccharides from strain ANU2840.

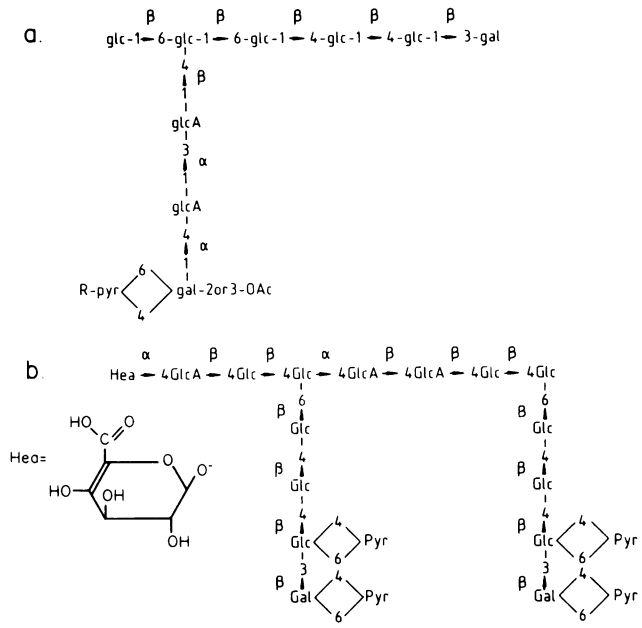

FIG. 2. Chemical structures of oligosaccharide repeat units. (A) Structure of the acidic oligosaccharide repeat unit of strain ANU280. (B) The structure of the acidic oligosaccharide repeat unit of strain ANU843. The 8-sugar repeat unit structure of *R. trifolii* NA30 and 0403 have been reported elsewhere (18).

TABLE 2. Mixing experiments on *Leucaena* plants

Inoculum strain(s)	Phenotype of inoculated plants[a]	No. of plants tested	% of plants forming nitrogen-fixing nodules
ANU265	Nod$^-$	14	0
ANU2811	Roots with calli	14	0
ANU2811 + ANU265	Fix$^+$ nodules	14	60
ANU2820	Roots with calli	12	0
ANU2820 + ANU265	Fix$^+$ nodules	12	60
ANU2840	Roots with calli	35	0
ANU2840 + ANU265	Fix$^+$ nodules	35	90
ANU845	Nod$^-$	12	0
ANU2840 + ANU845	Small Fix$^-$ nodules	12	0

[a] Plants with Fix$^+$ nodules reduce acetylene at approximately 20 to 40% of the efficiency of the parent strain, ANU280. Fix$^+$ nodules induced by strain ANU280 reduce 23 nmol of acetylene per mg of nodule (fresh weight) per h on *Leucaena* plants.

the essential nodulation genes, as well as the nitrogenase genes, have been deleted from this strain (16). The ^{13}C NMR spectrum of the isolated oligosaccharide repeat unit from strain ANU265 was indistinguishable from that for oligosaccharide from the parent strain ANU280.

When strain ANU265 was mixed in equal amounts with each of the Exo$^-$ mutants and coinoculated onto *Leucaena* plants; nitrogen-fixing nodules were produced (Table 2). Sections through these nodules showed a meristematic zone with well-formed vascular bundles and an extensive "bacteroid zone" containing rod-shaped nonswollen bacteria (Fig. 3). Both strain ANU265 and the Exo$^-$ mutants were isolated from the nitrogen-fixing nodules. The isolated bacteria retained their original colony morphology (Exo$^+$ for ANU265, and Exo$^-$ for the mutant strains), antibiotic resistance markers (Sp^r for ANU265 and Rf^r, Sm^r, and Km^r for the Exo$^-$ mutants), and nodulation phenotypes on *Leucaena* plants, indicating that no detectable genetic transfer had occurred between these strains. To determine whether the ANU265 background was important for the correction of Exo$^-$ mutants, the Sym plasmid-cured *R. trifolii* strain ANU845 (instead of ANU265) was mixed with the Exo$^-$ mutant ANU2840. Small nodules were formed rather than callus structures, but these were not nitrogen fixing (Table 2). Similarly, the addition of the heterologous purified EPS or oligosaccharide repeat unit isolated from strain ANU843 together with Exo$^-$ mutant ANU2840 onto *Leucaena* plants induced small nodules which failed to fix atmospheric nitrogen.

TABLE 3. Effect of adding purified EPS or nonasaccharide repeat unit on *Leucaena* nodulation[a]

Strain inoculated and addition	Plant nodulation response	No. of plants tested	% of plants forming Fix$^+$ nodules
ANU280 (parent strain)	Large Fix$^+$ nodules and occasional small calli	100	100
ANU2811	Small calli	100	2
ANU2840	Small calli	100	1
ANU2820	Small calli	20	0
ANU2811 + EPS from ANU280	Large Fix$^+$ nodules and small calli	30	35
ANU2811 + oligosaccharide repeat unit from ANU280	Large Fix$^+$ nodules and small calli	20	30
ANU2840 + EPS from ANU280	Large Fix$^+$ nodules and small calli	100	40
ANU2840 + oligosaccharide repeat unit from ANU280	Large Fix$^+$ nodules and small calli	20	40
ANU2820 + EPS from ANU280	Large Fix$^+$ nodules and small calli	17	25
ANU2820 + oligosaccharide repeat unit from ANU280	Large Fix$^+$ nodules and small calli	10	30

[a] Nitrogen-fixing nodules induced by the addition of EPS or oligosaccharide repeat unit and Exo$^-$ mutants on *Leucaena* reduce acetylene 30 to 80% as effectively as Fix$^+$ nodules induced by the parent strain, ANU280. Percentages of plants forming Fix$^+$ nodules are averages of 10-plant batch experiments. In each batch of 10 plants, the percentage of plants forming Fix$^+$ nodules varied from 30 to 80% with each Exo$^-$ mutant. EPS and oligosaccharide repeat unit were added 24 h prior to inoculation with Exo$^-$ bacteria.

Effect of adding purified EPS or related oligosaccharide and Exo$^-$ mutants to *Leucaena* plants. The acidic EPS from the parent strain ANU280 had been isolated and chemically sequenced (12), and the structure of the oligosaccharide repeat unit is shown in Fig. 2A. Purified EPS or related oligosaccharide isolated from strain ANU280 was inoculated onto *Leucaena* plants together with one of the Exo$^-$ mutants. This was done to determine whether the helper effect of strain ANU265 could be substituted for by the addition of purified EPS from the parent strain ANU280 or from the Sym plasmid-cured strain ANU265. In all cases, coinoculation of the EPS or the oligosaccharide repeat unit enabled the Exo$^-$ mutants to induce nitrogen-fixing nodules on *Leucaena* plants, although some calli were still produced (Fig. 3, Table 3). The same behavior was observed whether the EPS was obtained from ANU280 or ANU265. No Exo$^+$ bacteria were detected when the contents of 30 pigmented *Leucaena* nodules were analyzed, and the Exo$^-$ bacteria invariably retained their defective phenotype.

To measure the effect of EPS concentration, different amounts of purified EPS from ANU280 were added to plants with Exo$^-$ mutants ANU2811 and ANU2840. A standard volume of 10 μl was added to *Leucaena* seedlings at concentrations ranging from 0.1 to 10 mg/ml prior to the addition of the bacterial inoculum. Nodules, as distinct from callus-like structures, could be found on the lateral roots of *Leucaena* seedlings when as little as 1 μg of the EPS was added (Table 4).

TABLE 4. Effect of adding different amounts of EPS together with Exo$^-$ mutants ANU2811 and ANU2840 on *Leucaena* nodulation[a]

EPS added (μg)	No. of plants tested	% of plants forming Fix$^+$ nodules
100	80	35
50	50	40
5	20	40
1	20	30

[a] Fix$^+$ nodules induced by EPS or oligosaccharide repeat unit on *Leucaena* plants typically give 30 to 80% efficiency in acetylene reduction compared with nodules induced by the parent strain, ANU280.

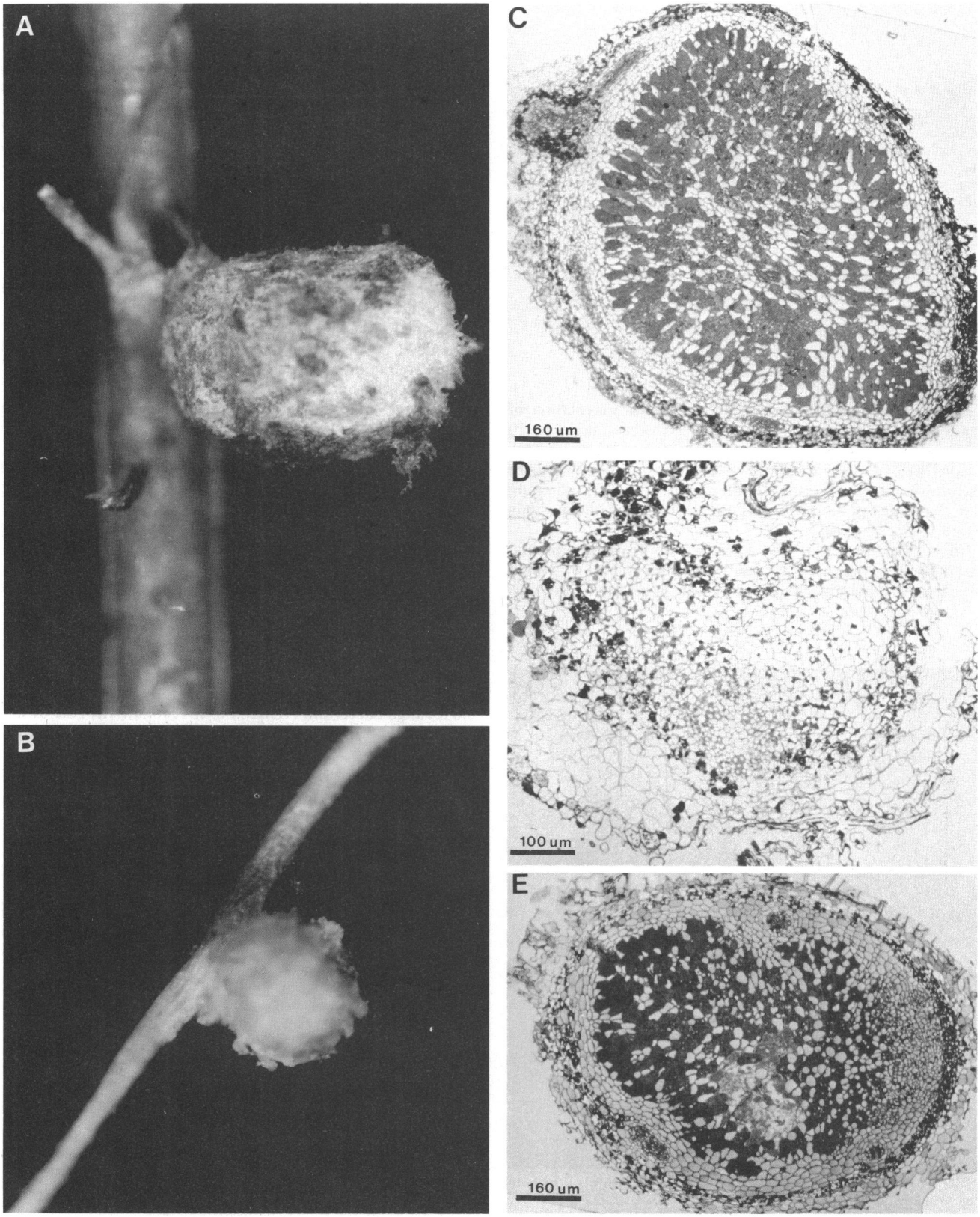

FIG. 3. Responses of *Leucaena plants* to the addition of EPS or oligosaccharide repeat unit with mucoid-defective strains. (A) A nitrogen-fixing (C_2H_2-reducing) nodule induced by the parent strain ANU280. (B) Non-nitrogen-fixing, distorted, calluslike nodules induced by the Exo^- mutant strain ANU2840. (C, D, and E) Ultrastructure of the nodules induced by ANU280 (C), the callus induced by strain ANU2840 (D), and the nodules induced by strain ANU2840 after the addition of EPS isolated from ANU280 (E).

TABLE 5. Effect of adding purified EPS or oligosaccharide repeat unit on siratro nodulation[a]

Strain inoculated and addition	Plant nodulation response	No. of plants tested	% of plants forming Fix+ nodules
ANU280 (parent strain)	Large Fix+ nodules	250	>90
ANU2811	Small Fix- nodules	80	2
ANU2820	Small Fix- nodules	20	0
ANU2811 + EPS from ANU280	Large Fix+ nodules	40	40
ANU2820 + EPS from ANU280	Large Fix+ nodules	30	40
ANU2820 + oligosaccharide repeat unit from ANU280	Large Fix+ nodules	20	40

[a] EPS and oligosaccharide repeat unit were added 24 h prior to inoculation of Exo- bacteria.

Effect of adding EPS before and after the inoculation of Exo- mutants. Portions (50 μg) of EPS were added to 10 *Leucaena* plants either (i) 24 h prior to, (ii) at the same time as, or (iii) 24 h after the Exo- bacteria were inoculated. In each case, nitrogen-fixing nodules were formed, indicating that the time of addition was not crucial for the periods tested.

Effect of adding EPS and Exo- strains to siratro plants. Parallel experiments were done on siratro plants, on which the parent strain induces nitrogen-fixing determinate nodules and the Exo- mutants ANU2811 and ANU2820 alone induce small nodules which fail to fix nitrogen. The addition of EPS from ANU280 24 h prior to inoculation with the Exo- strains ANU2811 and ANU2820 enabled these bacteria to produce determinate nitrogen-fixing nodules on siratro plants, like the parent strain (Table 5).

Addition of *R. trifolii* EPS or related oligosaccharide and *R. trifolii* Exo- mutant ANU437 to white clover. The second group of plants to be tested were clovers, which are temperate legumes. The structure of the EPS of *R. trifolii* ANU843 was determined by an approach similar to that used to determine the structure of the NGR234 EPS. *R. trifolii* ANU843 produces a 15-sugar modified oligosaccharide repeat unit (Fig. 2B). The Exo- *Rhizobium* sp. strain ANU437 is a Tn*5*-induced mutant derived from strain ANU794. Previous work has shown that strain ANU437 produces levels of EPS which are at least 1,000-fold lower than those produced by the parent strain and on clovers forms small, ineffective nodules which rapidly senesce (6).

To test whether the addition of the *R. trifolii* ANU843 EPS or related oligosaccharide could correct the nodulation-defective phenotype of the ANU437 Exo- mutant, 50 μg of either the *R. trifolii* EPS or related oligosaccharide was added to white clover seedlings 24 h prior to the inoculation of strain ANU437. After 5 weeks, 25% of the seedlings produced a proportion of nodules which resembled the pigmented nitrogen-fixing nodules produced by the parent strain (Fig. 4). These plants were able to reduce acetylene (Table 6). Bacteria isolated from these clover nodules were found to retain all the features of the inoculum strain ANU437.

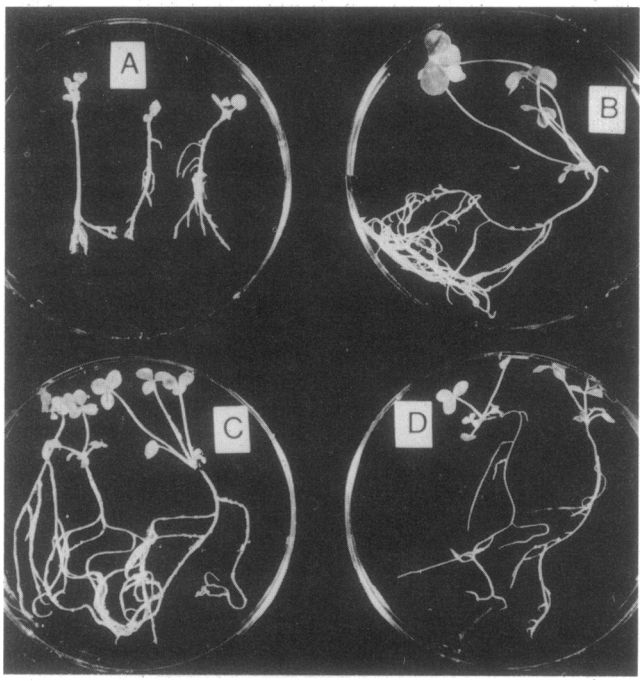

FIG. 4. Responses of white clover to the addition of EPS or oligosaccharide repeat unit with mucoid-defective strains. (A) White clover inoculated with the Exo- mutant ANU437. (B) White clover inoculated with the mucoid parent strain ANU794. (C) White clover with EPS added 16 h prior to inoculation with ANU437. (D) White clover with oligosaccharide repeat unit from ANU843 added 16 h prior to inoculation with ANU437. White clover seedlings inoculated with EPS alone did not nodulate.

DISCUSSION

We have used specific Tn*5*-induced mutants of the broad-host-range bacterium NGR234 to investigate the role of EPS on *Rhizobium* infection. The advantages of using these Tn*5*-generated strains are: (i) the defect is due to a single mutation; and (ii) the response of different plants to these strains can be assessed.

TABLE 6. Effect of adding EPS or oligosaccharide repeat unit isolated from *R. trifolii* ANU843 on nodulation of white clover by Exo- mutant ANU437[a]

Strain inoculated and addition	Plant nodulation response	No. of plants tested	% of plants forming Fix+ nodules
ANU794	Pigmented Fix+ nodules	>200	95
ANU437	Small Fix- nodules	>200	0
ANU437 + EPS from ANU843	Pigmented Fix+ nodules and small Fix- nodules	20	25
ANU437 + oligosaccharide repeat unit from ANU843	Pigmented Fix+ nodules and small Fix- nodules	20	30
ANU437 + EPS from ANU280	Small Fix- nodules	20	0
ANU437 + oligosaccharide repeat unit from ANU280	Small Fix- nodules	20	0

[a] EPS and oligosaccharide repeat unit were added 24 h prior to inoculation of Exo- mutant ANU437 to white clover seedlings.

Our results demonstrate unambiguously that restoration of the Fix^+ phenotype can be achieved by the addition of homologous EPS or oligosaccharide from the appropriate parent strain. The EPS may be provided by a mixed inoculation experiment. Heterologous saccharides failed to correct the defective symbiotic phenotype. Restoration of the the Fix^+ phenotype was successful with both indeterminate (*Leucaena*) and determinate (siratro) nodule-forming plants. Similarly, the defective nodulation properties of *R. trifolii* ANU437 were corrected only by addition of homologous EPS or oligosaccharide isolated from *R. trifolii* ANU843.

This specificity of action of saccharides indicates that they have more than a simple passive role of masking determinants on the *Rhizobium* surface. Recently, specific oligosaccharins derived from the cleavage of host or plant pathogen cell walls have been postulated to be regulators of specific plant functions, such as growth, differentiation, and disease resistance (8, 14). It is possible that the EPS or part of it functions similarly. The action of EPS may depend on the presence of plant enzymes (3, 10, 27) which degrade the bacterial polymer to active oligomers, which have a specific role in effective nodule formation.

The generality of the correction phenomenon remains to be demonstrated. While strains NGR234 and *R. trifolii* ANU843 are not closely related, there are some similarities in their symbiotic properties. Both are fast-growing strains that invade via root hair curling and infection thread formation. Both produce polysaccharides containing uronic acid. Exo^- mutants from both strains are able to initiate infection, but only to a stage of forming severely defective non-nitrogen-fixing nodules.

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