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Heterologous Exopolysaccharide Production in *Rhizobium* sp. Strain NGR234 and Consequences for Nodule Development

JAMES X. GRAY,[1] HANGJUN ZHAN,[2] STEVEN B. LEVERY,[3] LAURIE BATTISTI,[2] BARRY G. ROLFE,[1]* AND JOHN A. LEIGH[2]

Plant Microbe Interactions Group, Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra, A.C.T. 2601, Australia[1]; Department of Microbiology SC-42, University of Washington, Seattle, Washington 98195[2]; and The Biomembrane Institute, Seattle, Washington 98119[3]

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***Rhizobium* sp. strain NGR234 produces large amounts of acidic exopolysaccharide. Mutants that fail to synthesize this exopolysaccharide are also unable to nodulate the host plant *Leucaena leucocephala*. A hybrid strain of *Rhizobium* sp. strain NGR234 containing *exo* genes from *Rhizobium meliloti* was constructed. The background genetics and *nod* genes of *Rhizobium* sp. strain NGR234 are retained, but the cluster of genes involved in exopolysaccharide biosynthesis was deleted. These *exo* genes were replaced with genes required for the synthesis of succinoglycan exopolysaccharide from *R. meliloti*. As a result of the genetic manipulation, the ability of these hybrids to synthesize exopolysaccharide was restored, but the structure was that of succinoglycan and not that of *Rhizobium* sp. strain NGR234. The replacement genes were contained on a cosmid which encoded the entire known *R. meliloti exo* gene cluster, with the exception of *exoB*. Cosmids containing smaller portions of this *exo* gene cluster did not restore exopolysaccharide production. The presence of succinoglycan was indicated by staining with the fluorescent dye Calcofluor, proton nuclear magnetic resonance spectroscopy, and monosaccharide analysis. Although an NGR234 *exoY* mutant containing the *R. meliloti exo* genes produced multimers of the succinoglycan repeat unit, as does the wild-type *R. meliloti*, the deletion mutant of *Rhizobium* sp. strain NGR234 containing the *R. meliloti exo* genes produced only the monomer. The deletion mutant therefore appeared to lack a function that affects the multiplicity of succinoglycan produced in the *Rhizobium* sp. strain NGR234 background. Although these hybrid strains produced succinoglycan, they were still able to induce the development of an organized nodule structure on *L. leucocephala*. The resulting nodules did not fix nitrogen, but they did contain infection threads and bacteroids within plant cells. This clearly demonstrated that a heterologous acidic exopolysaccharide structure was sufficient to enable nodule development to proceed beyond the developmental barrier imposed on mutants of *Rhizobium* sp. strain NGR234 that are unable to synthesize any acidic exopolysaccharide.**

A complex interaction between rhizobia and specific legume plants results in the formation of nitrogen-fixing root nodules (17, 38, 45). The mechanisms of signal exchange and chemically based recognition between symbiotic partners are gradually being elucidated. For example, a small sulfated, *N*-acylated tetrasaccharide molecule that is synthesized by *Rhizobium meliloti* was shown to be a determinant of host range in the nodulation of alfalfa plants (35). In addition, it was demonstrated that *R. leguminosarum* bv. viciae could nodulate the heterologous host clover when the plants were transgenic for the pea root lectin, indicating an involvement of root lectins in determining host specificity (12). Lectins are carbohydrate-binding proteins, and it is known that legumes belonging to different cross-inoculation groups produce lectins with different oligosaccharide-binding specificities (27). A biological role for acidic exopolysaccharide (EPS) in *Rhizobium*-legume interactions was hypothesized because mutants of various *Rhizobium* species that are defective in EPS biosynthesis (Exo^-) are characterized by poor infectivity and nodule formation on plants on which the nodule ontogeny is indeterminate (5, 8, 9, 20, 34).

Rhizobium sp. strain NGR234 is capable of infecting a broad range of legume species. Exo^- mutants derived from this species induce poor nodulation on most plants. On the indeterminate nodulating legume *Leucaena leucocephala* only disorganized callus is formed; while this defect is not as severe as with *nod* mutants that form no root structures at all, the phenotype can still be considered Nod^-. Microscopic examination of the callus structures formed by Exo^- mutants on *L. leucocephala* reveals little or no bacterial penetration or colonization (9). The situation with Exo^- mutants of *Rhizobium* sp. strain NGR234 on *L. leucocephala* is in contrast to that of *R. meliloti* Exo^- mutants on alfalfa, in which organized nodules form. However, these alfalfa nodules also contain no bacteria. In general, Exo^- mutants can be functionally complemented by Exo^+ Nod^- mutants of the same wild-type strain when coinoculated on the host (6, 8, 11, 31, 41). In addition, some symbiotically defective Exo^- mutants of *Rhizobium* sp. strain NGR234, *R. leguminosarum* bv. trifolii, and *R. meliloti* are able to form nitrogen-fixing nodules on their respective hosts when inoculated in association with purified EPS and oligosaccharide repeat units isolated from their wild-type parental *Rhizobium* strains (2, 15). However, the addition of heterologous EPS did not correct the defective symbiotic phenotypes of the Exo^- mutants.

The structures of acidic EPS from *Rhizobium* sp. strain NGR234 and *R. meliloti* have a region of similarity, whereas the remainder of each molecule is unique. *Rhizobium* sp. strain NGR234 produces an EPS that has a nonasaccharide repeat unit which contains five glucoses, two galactoses, and two galacturonic acids, all in various α and β linkages, and one pyruvate and one acetate group (16). The oligosaccha-

* Corresponding author.

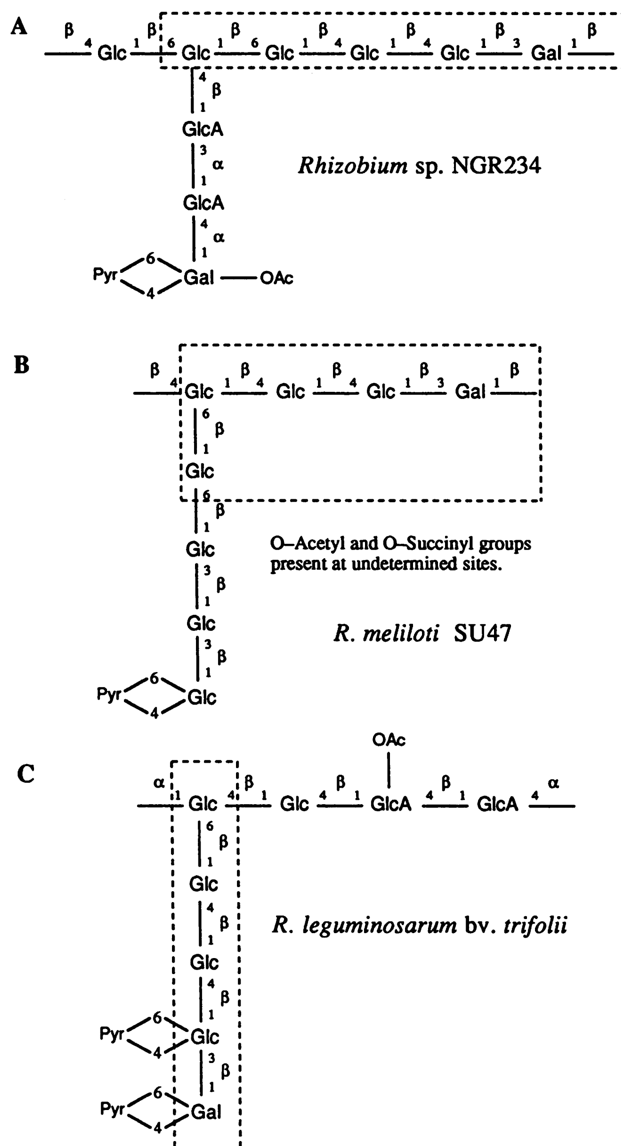

FIG. 1. Chemical structures of the oligosaccharide repeat units for the acidic EPS from *Rhizobium* sp. strain NGR234 (16) (A), *R. meliloti* succinoglycan (1) (B), and *R. leguminosarum* bv. trifolii (28) (C). The five sugars enclosed within boxes are regions of similarity between the three structures. Pyr, Pyruvate; OAc, O-acetyl.

ride repeat unit is structured so that it has a backbone of six sugars and a branched side chain of three sugars. In contrast, the major acidic EPS (succinoglycan) produced by *R. meliloti* SU47 is a polymer of octasaccharide repeat units that contain seven glucose units and one galactose, all in various β linkages, as well as one pyruvate, one acetate, and one succinate group (1). This repeat unit has a backbone of four sugars and a side chain of four sugars. Both oligosaccharide repeat units have a common carbohydrate region of one galactose and four glucoses, all with the same β linkages (Fig. 1A and B). Similarities also exist between the structures of EPS molecules of several other *Rhizobium* species. For example, the carbohydrate compositions of EPS from wild-type strains of *R. leguminosarum* biovars phaseoli, trifolii, and viciae have been shown to be identical, with the exception of some strains of *R. leguminosarum* bv. phaseoli, which differ only by the sugar composition of their side chains (40). However, the type and degree of noncarbohydrate substitutions of EPS molecules vary among *Rhizobium* species that otherwise have the same carbohydrate structure (42). The biological relevance of certain EPS structures is yet to be elucidated, although many suggestions implicate them in host range determination.

In both *Rhizobium* sp. strain NGR234 and *R. meliloti*, clusters of genes (*exo*) are required for the synthesis of acidic EPS and for nodule invasion and development on their respective hosts, alfalfa and *L. leucocephala*. Most of the *exo* genes in each species corresponded by hybridization and functional complementation to genes in the other species (54) and by DNA sequence comparisons (43). Some of these genes were responsible for posttranscriptional regulation of EPS synthesis and may form part of a membrane-bound catalytic-regulatory complex (24). The functional interchangeability of the *exo* genes between the two species allowed the construction of hybrid strains, which were used in this study to examine the effectiveness of *Rhizobium* strains that were synthesizing heterologous EPS molecules.

MATERIALS AND METHODS

Strains, plasmids, and media. Bacterial strains and plasmids used and constructed for this paper are listed in Table 1. *Rhizobium* sp. strain NGR234 strains were grown on BMM and TY as previously described (46). *R. meliloti* strains were grown on a modified LB or YM medium as previously described (54). Antibiotic concentrations for *Rhizobium* strains were 30 μg of rifampin per ml, 200 μg of kanamycin per ml, and 4 μg of tetracycline per ml.

Recombinant DNA techniques. DNA isolations, visualizations, and hybridizations were done by methods previously described (39). Hybond-N nylon membranes (Amersham, England) were used for DNA transfers. Restriction enzyme digests and ligations were done according to the specifications of the manufacturers (Boehringer Mannheim Biochemicals, Indianapolis, Ind., and New England BioLabs, Inc., Beverly, Mass.). DNA probes were ^{32}P-labeled with random primers (52).

Bacterial conjugation. Broad-host-range recombinant plasmids were mobilized from *Escherichia coli* NM522 into *Rhizobium* spp. by a triparental patch mating technique with pRK2013 (14) as a helper plasmid. *Rhizobium* sp. strain NGR234 transconjugants were selected as described previously (23). Complementing *R. meliloti* DNA cosmids were isolated as previously described (54).

Plant and acetylene reduction assays. Seed sterilization, germination, inoculation, and growth of *L. leucocephala* (Lamarck) deWit, var. Peru was described previously (9). Nodulated plants were tested for acetylene reduction by a method previously described (3).

Isolation of nodule bacteria. Nodules were cut from roots dipped into 100% ethanol, and then immersed in a drop of 1.25% sodium hypochlorite for 10 min to sterilize the surface. The surface-sterilized nodules were rinsed three times in sterile water and then crushed in a drop of protoplast dilution buffer (0.25 M sorbitol, 0.25 M mannitol, 2 mM $CaCl_2$) (25). The presence of bacteria within the crushed nodule tissue was detected on BMM agar.

Microscopic studies. Specimens were fixed in 2.5% glutaraldehyde and 3% formaldehyde in 30 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer, pH 6.8, for 12 h and then washed twice in 30 mM PIPES buffer (15 min each wash). The specimens were postfixed in 1% osmium tetrox-

TABLE 1. Bacterial strains and plasmids

Strain, plasmid, or phage	Description	Reference or source
R. meliloti strains		
SU47	Wild type	51
Rm1021	SU47 *str-21*	F. Ausubel
Rm1021ΔHKm	Rm1021 *exo* deletion	This work
Rm5000	SU47 *rif-5*	T. Finan
Rm7013	Rm5000 *exoB13*::Tn*5*	34
Rm7022	Rm1021Ω7022::Tn*5*, group E Exo$^-$	34
Rm7029	Rm1021Ω7029::Tn*5*, group E Exo$^-$	34
Rm7031	Rm1021 *exoA31*::Tn*5*	34
Rm7055	Rm1021 *exoF55*::Tn*5*	34
Rm8431	Rm1021 *exoL431*::Tn*5*	37
Rm8468	Rm1021 *exoP468*::Tn*5*	37
Rm8332	Rm1021 *exoQ332*::Tn*5*	37
Rhizobium sp. strain NGR234 derivatives		
NGR234	Wild-type broad-host-range cowpea *Rhizobium* sp.	48
ANU280	Sm^r Rif^r derivative of NGR234	9
ANU2811	ANU280 *exoY11*::Tn*5*	10, 23
ANU2820	ANU280 *exo* group A::Tn*5* (*exoB-C*)	10
ANU2822	ANU280 *exo* group C::Tn*5*	10
ANU2826	ANU280 *exo* group B::Tn*5*	10
ANU2840	ANU280 *exoY40*::Tn*5*	10, 23
ANU2871	ANU280 *exo* group D::Tn*5*	10
ANU616-d	Km^s, ANU2811 16-kb *exo* deletion	This work
E. coli strains		
HB101	*leu proA2 rps-120* (Sm^r) *hsdS20*	4
NM522	*recA*$^+$ [*supE thi* Δ(*lac-proAB*) *hsd-5* (F′ *proAB lacI*^{q} *lacZ*Δ*M15*)]	22
Plasmids and phage		
pD56	pLAFR1, *R. meliloti exoBF*-complementing plasmid	34
pEX154	pLAFR1, *R. meliloti exoAH*-complementing plasmid	33
pJG22	10-kb *Bam*HI fragment of wild-type *exo* DNA cloned into a broad-host vector, Tc^r	23
pJG40	pUC18 recombinant, 9-kb *Bam*HI fragment of wild-type *exo* DNA, Ap^r	This work
pRG100	pLAFR1, wild-type *R. meliloti exo* DNA, Tc^r	This work
pRG100ΔH	Deletion derivative of pRG100	This work
pRK2013	Helper plasmid, Tra$^+$ *oriT* ColE1 Km^r	14
pUC18	*E. coli* specific, *lac′Z* Ap^r	50
R′3222	Wild-type *Rhizobium* sp. strain NGR234 *exo* DNA, Tc^r	10
φM12	Generalized transducing phage for *R. meliloti* SU47	T. Finan

ide in 30 mM PIPES buffer for 2 h, rinsed in distilled water, immersed in 0.5% uranyl acetate for 45 min, and washed in distilled water. The specimens were dehydrated in a gradual acetone series and then prepared for embedding in Spurr's resin by gradually increasing the ratio of acetone to Spurr's resin from 1:1 to 1:4 over 48 h. Finally, the specimens were embedded in fresh resin that was polymerized overnight at 65°C. Sections for light microscopy were cut to 0.5 μm with a glass knife, stained with toluidine blue, pH 11.1, and viewed with a Nikon Optiphot and Plan Apo objectives. Sections for electron microscopy were cut to 70 to 90 nm with a diamond knife, mounted onto Formvar-coated slot grids, and stained for 7 min with 2% uranyl acetate in ethanol and then for 5 min with lead citrate (44). Electron microscopic sections were viewed under a Jeol 2000Ex microscope.

Preparation and analysis of polysaccharide. Identification of β-(1,2)-glucan by proton nuclear magnetic resonance (NMR) spectroscopy and analysis of lipopolysaccharide (LPS) by gel electrophoresis were done as previously described (32). Calcofluor staining of colonies was performed as described previously (34).

EPS samples were obtained in several ways, as described previously (32). Briefly, the simplest preparations were obtained by the dialysis of culture supernatants. High-molecular-weight (HMW) and low-molecular-weight (LMW) fractions of EPS were obtained by Biogel A5 chromatography of culture supernatants. HMW EPS invariably eluted in the void volume of the column, representing a molecular mass on the order of 5 million Da, while LMW EPS eluted in the salt volume and therefore had a molecular mass less than 10,000 Da. HMW EPS was then desalted by dialysis, and LMW EPS was desalted by Biogel P2 chromatography. LMW EPS could sometimes be further fractioned by Biogel P4 chromatography as previously described (47). This technique separated LMW succinoglycan into distinct fractions that were identified as monomer, trimer, and tetramer of the repeat unit (2a).

Monosaccharide compositions of EPS samples were determined by gas chromatography-mass spectrometry analysis of per-*O*-trimethylsilyated methyl glycosides after methanolysis (36). Response factors were adjusted to give correct compositions for standard EPS samples in terms of total galactose, glucose, and glucuronic acid (GlcA). Complete

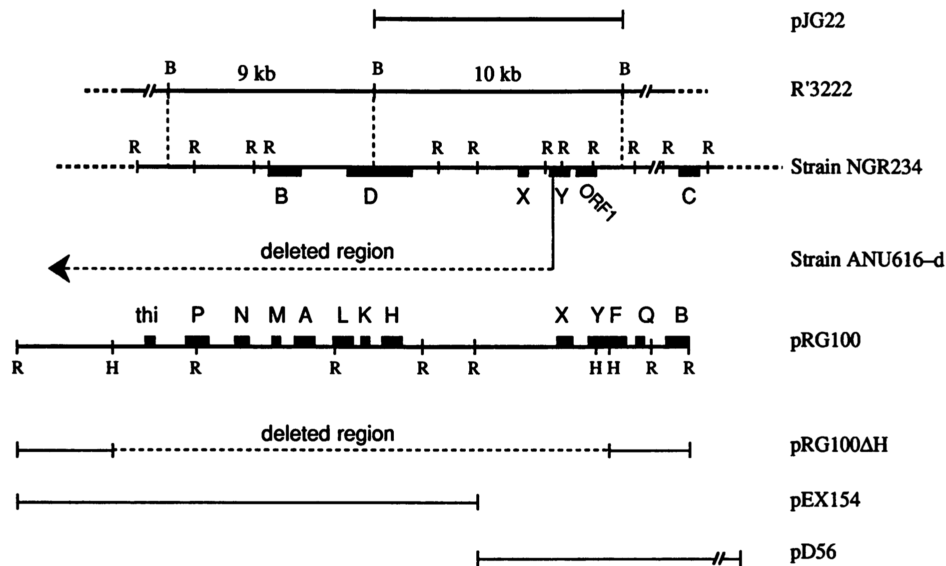

FIG. 2. Schematic of the various *Rhizobium* sp. strain NGR234 and *R. meliloti* constructs. The region of *exo* DNA deleted from the *Rhizobium* sp. strain NGR234 genome to create strain ANU616-d is indicated. The extent of *Rhizobium* sp. strain NGR234 DNA covered by plasmids R'3222 and pJG22, and also the *R. meliloti* DNA carried by pRG100 and other cosmids, is shown. The region of *R. meliloti exo* DNA deleted to create pRG100ΔH and subsequently Rm1021ΔHKm is shown. Restriction enzyme sites: B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI. ORF, Open reading frame.

pyruvylation of one hexose residue in each repeating unit of the standards was not assumed, but it was partitioned according to relative integrated peak areas within each hexose type. In experimental samples, the proportions of pyruvylated hexoses were calculated by adding the relative molar response of the pyruvylated hexose to the corresponding nonpyruvylated hexose, calculating the correct total of each hexose, and then reapportioning the amount of pyruvylated hexose according to the area relative to nonpyruvylated hexose.

RESULTS

Isolation and characterization of pRG100. In our previous study (54), most of the *Rhizobium* sp. strain NGR234 *exo* mutants could be complemented by cosmid clones of the *R. meliloti exo* region. However, we failed to complement ANU2826, an Exo$^-$ group B mutant of *Rhizobium* sp. strain NGR234. In an attempt to find complementing genes from *R. meliloti*, a genomic clone bank from *R. meliloti* Rm1021 was mobilized into mutant strain ANU2826 and mucoid colonies were selected from yeast-mannitol (YM) agar plates by previously described techniques (55). Four mucoid colonies were isolated, and the cosmid DNA from each of these isolates showed identical restriction patterns and were designated pRG100. Cosmid pRG100 has the same restriction profile as the previously reported cosmid, pEX312 (37), and carries all the known *exo* genes of the *R. meliloti exo* region except *exoB*. Thus, as expected from the genetic map (Fig. 2), pRG100 complemented *R. meliloti exoP*, *exoA*, *exoL*, *exoF*, and *exoQ*. It did not complement *exoB*, apparently because it contains only part of this gene.

Construction of deletion strains of *Rhizobium* sp. NGR234. Strain ANU2811 is an *exoY*::Tn*5* mutant of strain ANU280 (a wild-type derivative of *Rhizobium* sp. strain NGR234) that fails to synthesize acidic exopolysaccharide (Exo$^-$) or its repeat unit and is also unable to nodulate *L. leucocephala* (except for callus formation, Nod$^-$), which is a symbiotic host for strain ANU280 (9). Introduction of a recombinant plasmid (pJG22) carrying wild-type DNA (*exoX exoY*) corresponding to the mutated region will restore an Exo$^+$ phenotype to only 52% of the transconjugants (23). The remaining transconjugants were shown to have undergone a double reciprocal recombination event that resulted in the replacement of the *exoY* wild-type DNA on the plasmid with the *exoY*::Tn*5* mutant allele from the chromosome, and as a consequence of the *exoX* gene dosage remaining high relative to that of *exoY*, the colonies remained Exo$^-$ (23). A small percentage of these Exo$^-$ transconjugants could be cured of their IncP1 plasmids by screening for loss of the plasmid antibiotic marker (tetracycline); in every such case, the Tn*5* antibiotic marker (kanamycin) was also lost, since both antibiotic markers were now plasmid borne because of the recombination events. It was anticipated that these plasmid-cured strains would be Exo$^+$ and equivalent to the wild-type strain, but the colony phenotypes unexpectedly remained Exo$^-$.

To investigate the *exo* DNA region at the molecular level, genomic DNA was isolated from the plasmid-cured strains and examined for sequences hybridizing to ^{32}P-labeled probes for the region. First, none of the strains showed any hybridization to vector sequences. Second, hybridization of the strains to a 10-kb *Bam*HI fragment of *exo* DNA demonstrated the existence of large deletions in the Exo$^-$ strains (Fig. 3). All *Eco*RI fragments to the right (as shown in Fig. 2) of the 2811::Tn*5* insertion fragment were present, while all those to the left were absent. In addition, there was no hybridization to another 9-kb *Bam*HI DNA fragment (Fig. 3), which is the adjacent fragment to the left of the first 10-kb *Bam*HI fragment (Fig. 2). This result indicated that the Exo$^-$ strains have suffered deletions that extend leftwards (Fig. 2) from the 2811::Tn*5* insertion site and involve at least 16 kb of DNA. The deletion began within the *exoY* coding region (presumably at the Tn*5* insertion site) and has effectively removed the *exoY* operon, *exoX*, and genetic complementa-

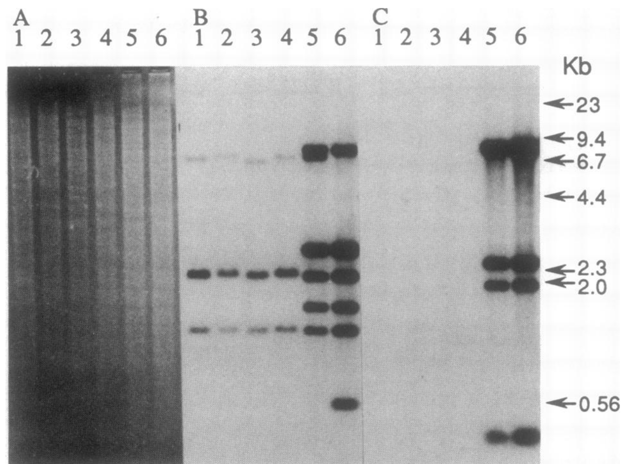

FIG. 3. Analysis of genomic DNA from deletion mutant strains of *Rhizobium* sp. strain NGR234. All DNA has been restricted with *Eco*RI. Lanes 1 to 4, DNA from separate isolates of Exo$^-$ deletion mutants; lane 5, DNA from *exoY*::Tn*5* mutant strain ANU2811; lane 6, DNA from the wild-type strain ANU280. (A) DNA electrophoresed through a 0.7% agarose gel; (B and C) autoradiographs of a Southern blot that was probed with ^{32}P-labeled 10-kb *Bam*HI DNA from pJG22 (B) and with ^{32}P-labeled 9-kb *Bam*HI DNA to the left (Fig. 2) of the 10-kb *Bam*HI fragment (C).

tion groups B and D, which have all been shown to be clustered in the *Rhizobium* sp. strain NGR234 genome (10). One of these deletion mutants was named strain ANU616-d and was used for further analysis.

The deleted region does not include genes required for LPS or β-(1,2)-glucan synthesis. The deletion mutant strain ANU616-d did not produce any detectable acidic EPS, but its production of LPS and β-(1,2)-glucan was not affected. This was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of LPS from wild-type strain ANU280 and mutant strain ANU616-d, which revealed the same banding profile (not shown). In addition, the culture supernatant and ethanol-water extracts of ANU616-d and ANU280 cultures yielded carbohydrate material with proton NMR spectra similar to that of β-(1,2)-glucan. Thus, the region of DNA deleted in strain ANU616-d definitely encodes genes involved in acidic EPS production but does not appear to be involved in the synthesis of any other extracellular polysaccharide molecules.

Restoration of EPS biosynthesis by the introduction of cloned DNA. A very large R-prime plasmid, R′3222 (10), which carries approximately 65 kb of wild-type *Rhizobium* sp. strain NGR234 *exo* DNA, was able to restore EPS synthesis to strain ANU616-d(R′3222) transconjugants, whereas the smaller pJG22 plasmid, which carries only a 10-kb DNA fragment from this region (Fig. 2), was unable to restore EPS synthesis to ANU616-d(pJG22) transconjugants. In addition, the cosmid pRG100, which carries *R. meliloti exo* genes, was able to fully restore an Exo$^+$ colony morphology to strain ANU616-d(pRG100) transconjugants. Interestingly, ANU616-d(pRG100) colonies were transiently fluorescent for the first few days when grown on agar plates containing Calcofluor. Since succinoglycan is Calcofluor stainable and the EPS produced by *Rhizobium* sp. strain NGR234 is not, it was apparent that a succinoglycanlike EPS was being produced by ANU616-d(pRG100) transconjugants.

Cosmid pRG100 was mobilized into other Exo$^-$ mutant backgrounds of *Rhizobium* sp. strain NGR234 to test whether the Calcofluor-stainable EPS could be produced. Colonies of ANU280 (Exo$^+$ wild type), ANU2826 (group B Exo$^-$), ANU2871 (group D Exo$^-$), ANU2840 (*exoY40*::Tn*5*), and ANU2811 (*exoY11*::Tn*5*), all containing pRG100, were all mucoid and fluorescent (Calcofluor bright) on YM agar plates with Calcofluor. As expected, colonies of ANU2820 (group B and C double mutant) and ANU2822 (*exoC*) containing pRG100 were Exo$^-$ and nonfluorescent (Calcofluor dark), since *R. meliloti exoB* or its counterpart *Rhizobium* sp. strain NGR234 *exoC* would be required for the synthesis of EPS.

Characterization of the heterologous EPS. To confirm that Calcofluor staining was a true indicator of the production of a succinoglycanlike EPS in the *Rhizobium* sp. strain NGR234 background, EPS samples of ANU2826 (group B), ANU2871 (group D), and ANU616-d, all containing pRG100, were obtained by the dialysis of liquid culture supernatants and analyzed by proton NMR spectroscopy. The spectra (Fig. 4) showed the clear presence of succinate as well as other peaks characteristic of succinoglycan. Peaks characteristic of the *Rhizobium* sp. strain NGR234 EPS were also present in the spectrum of ANU2871(pRG100); this was expected because of complementation of *Rhizobium* sp. strain NGR234 *exoD* by functionally equivalent *R. meliloti* genes present on pRG100 (54). Peaks characteristic of the *Rhizobium* sp. strain NGR234 EPS may also be present in the spectra of ANU2826(pRG100), although less discernibly. Also evident in the samples from ANU2826(pRG100) and ANU616-d(pRG100) is β-(1,2)-glucan, a normal secretion product not affected by most *exo* mutations.

An analysis of sugar compositions of the EPS samples, performed on LMW EPS fractions (Table 2), confirmed the production of succinoglycan by the chimeric strains. Distinguishing characteristics of succinoglycan (Fig. 1B) are a total Glc:Gal ratio of 7:1 and the presence of pyruvylated glucose. Distinguishing characteristics of the *Rhizobium* sp. strain NGR234 EPS (Fig. 1A) are a total Glc:Gal ratio of 5:2, the presence of pyruvylated galactose, and the presence of GlcA. When EPS samples from wild-type strains were used as standards, the observed compositions of complemented constructs were very close to those of the standard samples (Table 2). Thus, *R. meliloti exoF* mutants containing a *Rhizobium* sp. strain NGR234 R-prime plasmid that complements *exoF* (54) produced succinoglycan, and the *Rhizobium* sp. strain NGR234 deletion mutant containing a complementing R-prime produced the *Rhizobium* sp. strain NGR234 EPS. When the same analysis was applied to LMW EPS from ANU2826(pRG100) and ANU616-d(pRG100), the absence of GlcA and the presence of pyruvylated Glc (Table 2) suggested that these samples were succinoglycan with no *Rhizobium* sp. strain NGR234 EPS. However, the presence of pyruvylated Gal (Table 2) is not consistent with the normal form of succinoglycan. It might indicate a new form of succinoglycan, perhaps pyruvylated at the reducing galactose. In these last two samples, the Glc:Gal ratios are not useful because of the presence of unknown quantities of β-(1,2)-glucan.

Multiplicity of succinoglycan produced in *Rhizobium* sp. strain NGR234 backgrounds. We have found that in *R. meliloti*, LMW succinoglycan consists of different multimers (monomer, trimer, and tetramer) of the repeat unit (2a). The LMW succinoglycan can be fractionated by Biogel P4 sizing chromatography into three distinct peaks that represent the different multiplicities of the repeat unit. To test whether

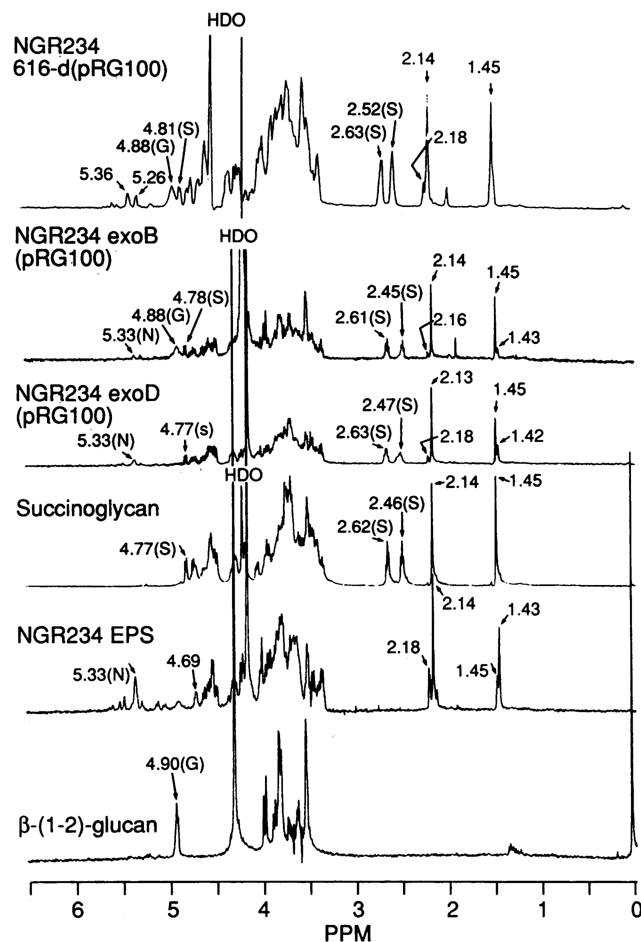

FIG. 4. Proton NMR spectra of EPSs prepared by dialysis of supernatants. The chemical shifts of signals characteristic of certain EPSs are shown beside the peaks. S, N, and G denote peaks characteristic of succinoglycan, strain NGR234 EPS, and β-(1,2)-glucan, respectively. The identities of selected peaks are as follows: 5.33 ppm, α-anomeric protons of Gal or GlcA at the nonreducing terminus of the *Rhizobium* sp. strain NGR234 EPS; 4.90 ppm, β-anomeric protons of β-(1,2)-glucan; 4.77 ppm, a β-anomeric proton of succinoglycan; 2.62 and 2.46 ppm, succinyl modifications; 2.14 ppm and vicinity, acetyl groups; 1.45 ppm and vicinity, pyruvyl groups.

LMW succinoglycan produced in *Rhizobium* sp. strain NGR234 backgrounds also contained multimeric forms, we subjected LMW EPS samples to P4 chromatography. Strain ANU2811 (*exoY11*::Tn*5*) containing pRG100 produced mainly trimers and tetramers. In contrast, strain ANU616-d(pRG100) produced only monomers. Therefore, the deletion in ANU616-d appears to have eliminated a function that can act on the heterologous EPS succinoglycan and controls the multiplicity of the product. Consistent with this idea, groups B and D and *exoY* mutants of *Rhizobium* sp. strain NGR234 containing pRG100 all produced an HMW EPS fraction that produced a fluorescent spot when dropped onto an agar plate containing Calcofluor. In contrast, ANU616-d(pRG100) produced no distinct HMW peak.

Symbiotic effectiveness of the deletion mutant and transconjugants. The deletion mutant, strain ANU616-d, formed non-nitrogen-fixing calli on the roots of *L. leucocephala* which were indistinguishable from those produced by Tn*5*-generated Exo⁻ mutants. These calli were small growths on the root surface (Fig. 5B) which did not reduce acetylene, and the plants clearly did not fix nitrogen (Fig. 5D). Light microscopic examination of the calli showed no obvious meristematic zone, and there was a general lack of the cellular organization that is present in normal nodules (Fig. 6A and B). Electron microscopic examination showed an absence of infection thread formation and no bacteria within plant cells, which correlated with the fact that rhizobia could not be recovered from crushed calli. In addition, the presence of darkly stained inclusions are signs of probable plant cell death, similar to hypersensitive reactions induced by invading fungi on some plants (9, 13).

TABLE 2. Sugar analysis of EPSs[a]

Strain	Molar proportion[b]						
	Gal + P-Gal	Gal	P-Gal	Glc + P-Glc	Glc	P-Glc	GlcA
Rm1021 (std)[c]	1.0	1.0	ND[d]	7.0	6.6	0.4	ND
Rm1021*exoF*(R'3222)	1.1	1.1	ND	6.9	6.3	0.6	ND
ANU280 (std)	2.0	1.2	0.8	5.0	5.0	ND	2.0
ANU616-d(R'3222)	2.1	1.0	1.1	4.9	4.9	ND	2.0
ANU280*exoB*(pRG100)	0.4	0.4	0.1	7.0	6.9	0.1	ND
ANU616-d(pRG100)	0.9	0.6	0.3	6.3	6.2	0.1	ND
β-(1,2)-glucan (std)	ND	ND	ND	1.0	1.0	ND	ND

[a] All EPSs were LMW samples from A5 chromatography except β-(1,2)-glucan, which was a cell extract (see Materials and Methods).
[b] P-Gal, Pyruvylated galactose; P-Glc, pyruvylated glucose.
[c] std, Standard (see Materials and Methods).
[d] ND, Not detected.

The transconjugant strains ANU616-d(R'3222) and ANU616-d(pRG100) had similar symbiotic phenotypes. They were able to invade plant cells and induce non-nitrogen-fixing nodules on the roots of *L. leucocephala*. These nodules were smaller than those produced by the wild type (Fig. 5A and C); they were unable to reduce acetylene, and the growth of the *L. leucocephala* plants was similar to that of uninoculated control plants (Fig. 5D). Microscopic examination of sections from the Fix⁻ nodules induced by transconjugant strains ANU616-d(R'3222) and ANU616-d(pRG100) revealed an organized nodule structure (Fig. 6C). There was evidence of a meristematic zone, infection threads, and a high frequency of plant cells infected by many bacteria (Fig. 6E). Virtually all of the bacteria had large white inclusions, which is indicative of β-hydroxybutyrate accumulation (18, 26).

Transconjugant strains ANU616-d(R'3222) and ANU616-d(pRG100) also formed Fix⁻ nodules on *Macroptilium atropurpureum* (sirato), a determinate nodulating host plant for *Rhizobium* sp. strain NGR234 (data not shown). Alfalfa inoculated with ANU616-d(pRG100) showed no response, including an absence of root hair curling.

EPS synthesis in various *R. meliloti* backgrounds. The converse of the above, i.e., the production of *Rhizobium* sp. strain NGR234 heterologous EPS in *R. meliloti*, was not observed. The R-prime plasmid R'3222 contains a cluster of *Rhizobium* sp. strain NGR234 *exo* genes and complements many *R. meliloti exo* mutants, including *exoF*. Proton NMR spectroscopy (54) and monosaccharide analysis (Table 2) indicated that only succinoglycan was produced by *R. meliloti exoF* mutants containing R'3222. In case the production of succinoglycan somehow inhibits the synthesis of the NGR234 EPS, perhaps by depleting precursors or saturating parts of the EPS biosynthetic apparatus, we tested

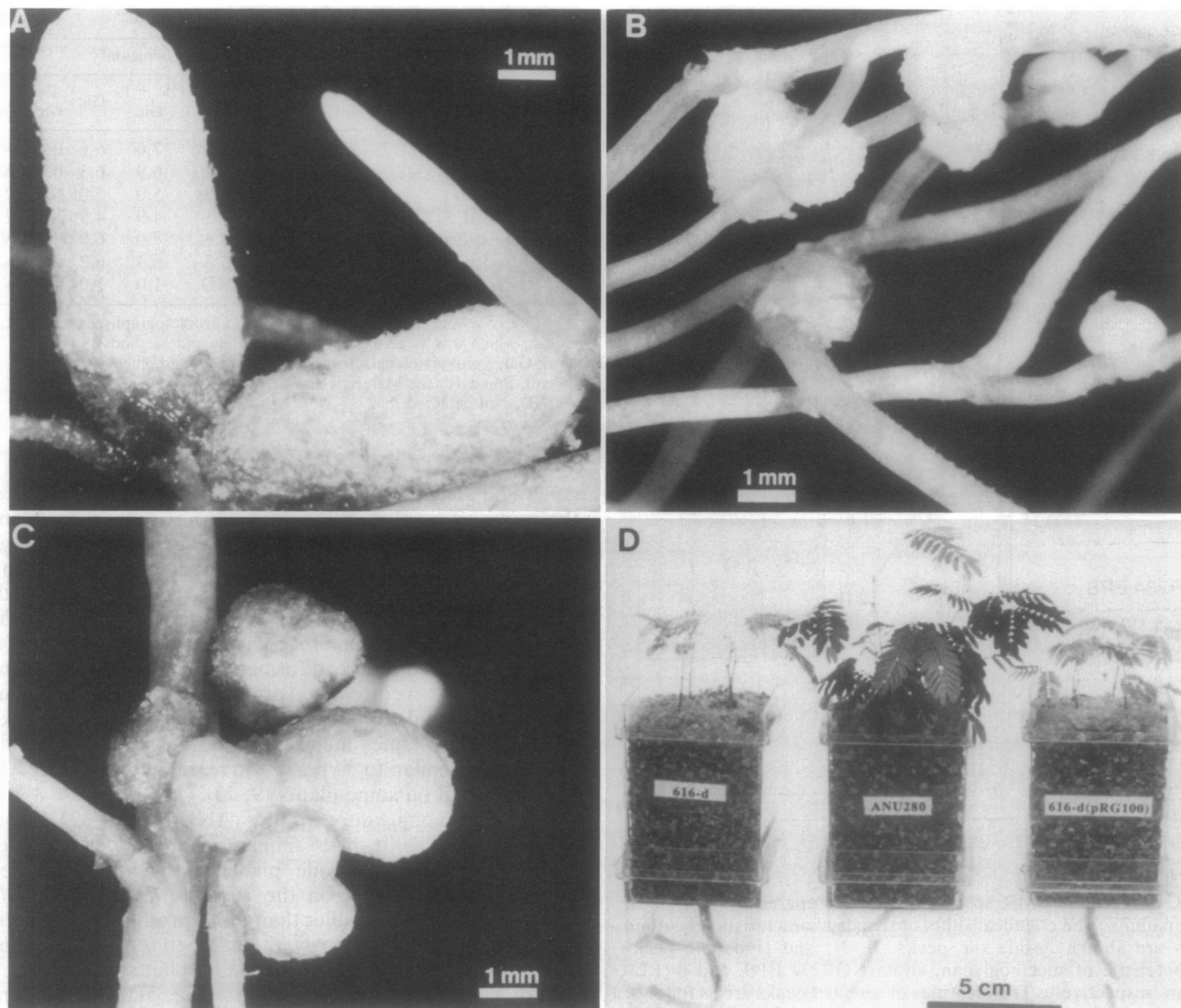

FIG. 5. Comparison of nodule and callus structures and symbiotic efficiencies for various *Rhizobium* constructs on *L. leucocephala*. (A) Wild-type strain ANU280 forms Fix^+ nodules; (B) deletion strain ANU616-d forms calli; (C) transconjugant strain ANU616-d(pRG100) forms Fix^- nodules. The structures were photographed from 5-week-old *L. leucocephala* plants. (D) Comparative growth of 7-week-old *L. leucocephala* plants inoculated with indicated strains.

whether deleting large portions of the *R. meliloti exo* region would eliminate succinoglycan production and allow the production of the NGR234 EPS in the presence of R'3222. The group E *exo* mutants of *R. meliloti* were tested first, using strains Rm7022 and Rm7029, which contain deletions of the entire known *exo* cluster (19). Transconjugants Rm7022(R'3222) and Rm7029(R'3222) were both nonmucoid and Calcofluor dark on YM agar, indicating that neither EPS was produced.

In a further effort to demonstrate heterologous EPS production in *R. meliloti*, a smaller deletion of the *R. meliloti exo* region was constructed. We wanted to make a deletion that was the approximate counterpart of the *Rhizobium* sp. strain NGR234 deletion in ANU616-d, which allows succinoglycan production in the *Rhizobium* sp. strain NGR234 background (see above). A 17-kb *Hin*dIII fragment was deleted from pRG100 (Fig. 2) and replaced with a 3.2-kb *Hin*dIII fragment from Tn*5* that encodes kanamycin resistance (29). The resultant plasmid was mobilized into *R. meliloti* Rm1021 and homogenotized into the genome. Finally, the marker was transduced into strain Rm1021 to give the *exo* deletion strain Rm1021ΔHKm. In confirmation of the identity of the deletion strain, neither pEX154 nor pD56, recombinant cosmids that would cover only parts of the deleted fragment (Fig. 2), restored Calcofluor staining, and *Hin*dIII-digested genomic DNA from Rm1021ΔHKm lacked sequences that hybridized to the appropriate *Hin*dIII fragment of pRG100. The deletion removed all of the *exo* genes from *exoP* to *exoYF* inclusive. It also appeared to eliminate the expression of *exoQ*, since pRG100ΔHKm failed to complement an *exoQ* mutant. Recent data from DNA sequencing indicates that *exoY*, *exoF*, and *exoQ* belong to the same transcription unit (30). Most of our insertions previously referred to as *exoF* lie within the *exoF* open reading frame, except *exoF210*::Tn*5* (54), which probably lies in *exoY*. Cosmid pRG100 complemented mutant strain Rm1021ΔHKm, as expected.

Transconjugant Rm1021ΔHKm(R'3222) colonies were

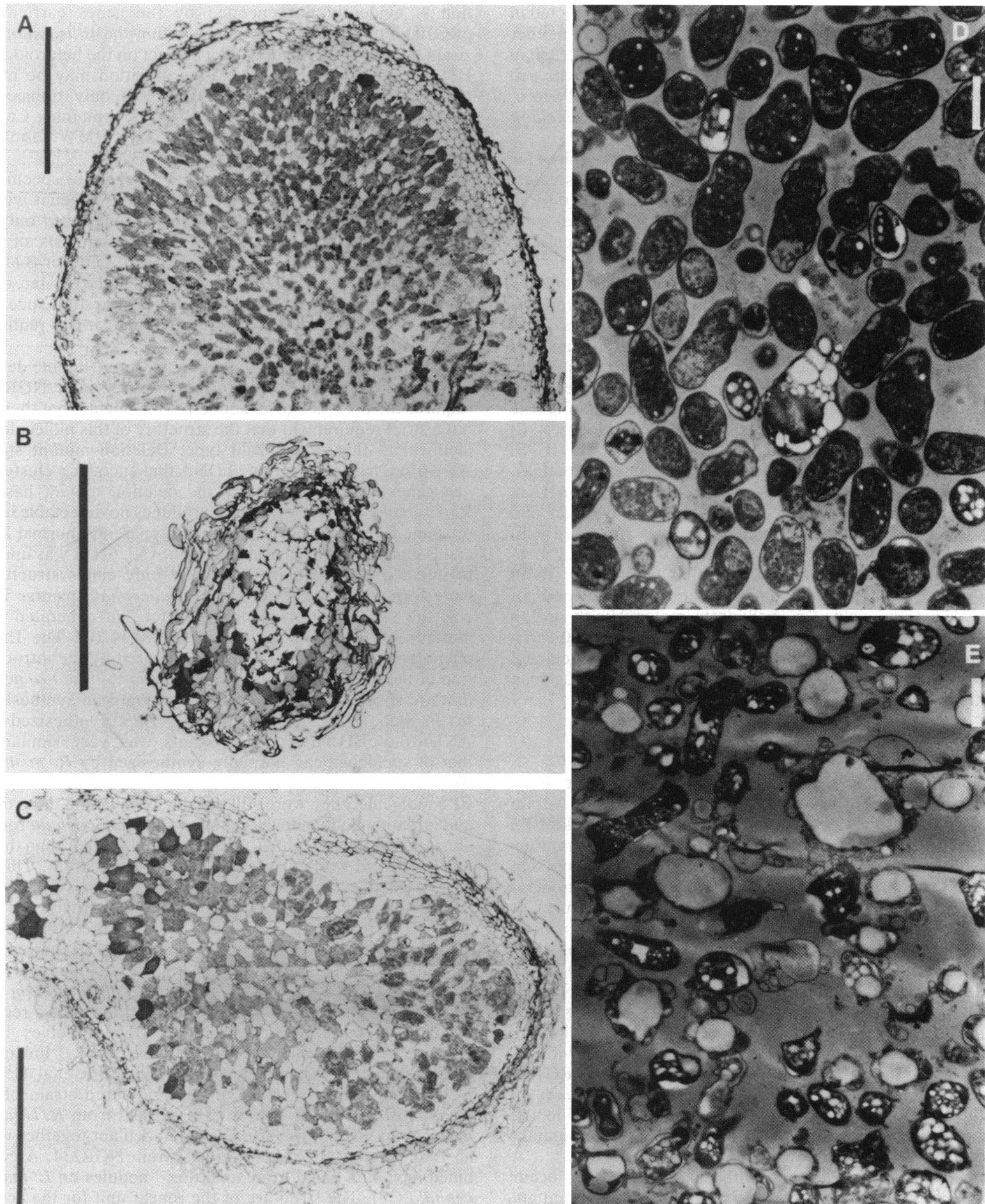

FIG. 6. Ultrastructural analysis of nodule and callus structures from *L. leucocephala* plants. (A) Part of a longitudinal section through a nitrogen-fixing nodule induced by the wild-type strain ANU280; (B) a longitudinal section through a callus structure induced by the deletion strain ANU616-d; (C) a longitudinal section through a Fix$^-$ nodule induced by the transconjugant strain ANU616-d(pRG100); (D) wild-type ANU280 bacteroids within an infected plant cell of a nitrogen-fixing nodule; (E) transconjugant ANU616-d(pRG100) bacteroids within an infected plant cell of a Fix$^-$ nodule. The bars in the light microscopic sections (A, B, and C) represent 0.5 mm, and the bars in the electron microscopic sections (D and E) represent 1 μm.

nonmucoid and Calcofluor dark, and no EPS was detected in liquid culture after 6 days of incubation. Since neither NGR234 EPS nor succinoglycan was produced, R'3222 may not carry (or may not express in *R. meliloti*) all of the *exo* genes specific for *Rhizobium* sp. strain NGR234 EPS production and therefore may not complement all the *R. meliloti* genes, known or unknown, between *exoP* and *exoQ*.

Genes between *exoP* and *exoQ* inclusive are not required for EPSb, LPS, or β-(1,2)-glucan synthesis. In previous studies, most *R. meliloti exo* mutants tested were deficient only in succinoglycan synthesis; that is, they were normal with respect to EPSb (EPS-II) (21, 55), LPS, and the periplasmic β-(1,2)-glucan. However, *exoB* mutants were defective in EPSb and LPS synthesis, and *exoC* (a gene not in the *exo* cluster) mutants were defective in all three carbohydrates as well as succinoglycan. It was recently shown that the *exoB* gene is required for galactose epimerase activity (7) and the *exoC* gene is required for phosphoglucomutase activity (49). In addition, Glazebrook and Walker (21) reported that EPS-II was synthesized in a strain with a deletion of all the *exo* genes except *exoB*. Our results were as follows: (i) mutant strain Rm1021ΔHKm did not synthesize any EPS; (ii) the introduction of the recombinant cosmid pMuc (55), containing genes for EPSb synthesis, into Rm1021ΔHKm yielded mucoid, Calcofluor dark colonies on LB agar and YM agar plates, indicating that EPSb was produced; (iii) SDS-PAGE of Rm1021 LPS and Rm1021ΔHKm LPS revealed the same banding patterns (not shown); (iv) both culture supernatant and ethanol-water extracts of cells of Rm1021ΔHKm yielded carbohydrate material with proton NMR spectra similar to the spectrum of authentic β-(1,2)-glucan (Fig. 4). Therefore, none of the genes in the deleted region have any known function other than in succinoglycan synthesis.

DISCUSSION

We have shown that the transfer of a large set of *R. meliloti exo* genes into derivatives of *Rhizobium* sp. strain NGR234 results in the production of an *R. meliloti*-like EPS by the *Rhizobium* sp. strain NGR234 transconjugants. Furthermore, the following three lines of evidence indicated that the EPS produced was similar if not identical to succinoglycan: staining by Calcofluor, succinoglycanlike features of proton NMR spectra, and monosaccharide compositions indicative of succinoglycan. Succinoglycan synthesis occurred when the cosmid transferred was pRG100, which contains all of the known genes of the *exo* cluster except *exoB*, but not when cosmids carrying smaller portions of the *R. meliloti exo* region were introduced. Therefore, all of the genes required for specific steps in succinoglycan synthesis appeared to lie on the 25-kb fragment cloned in pRG100. Succinoglycan production did not require any *Rhizobium* sp. strain NGR234 genes within the region from *exoY* to the group B Tn*5* insertion sites, since the deletion mutant ANU616-d also supported succinoglycan production.

Although succinoglycan synthesis appeared to occur regardless of the *Rhizobium* sp. strain NGR234 mutant background, the multiplicity of the succinoglycan repeat unit differed in mutants in individual *exo* genes compared with the deletion mutant. For Tn*5*-generated mutants, succinoglycan multimers within the LMW fraction were present, as was HMW succinoglycan, but only monomeric succinoglycan could be detected in the deletion mutant. These results are suggestive of the absence of a polymerization function in the deletion mutant background. The polymerization function is evidently not encoded by the genes carried on pRG100 but is encoded by genes within the *Rhizobium* sp. strain NGR234 *exo* region, and it can act on the heterologous EPS succinoglycan. However, the situation may be more complex, since ANU616-d(pRG100) was only transiently fluorescent when growing on agar plates containing Calcofluor. Previously, it was found that only HMW forms of succinoglycan are Calcofluor stainable (32).

We were also able to demonstrate a degree of specificity within *R. meliloti* by showing that all of the *exo* genes within the region from *exoP* to *exoQ* inclusive functioned only in succinoglycan synthesis and not in the synthesis of the second *R. meliloti* EPS (EPSb or EPS II), LPS, or β-(1,2)-glucan. Therefore, of all the known genes in the cluster, only *exoB* is required for other carbohydrates as well as succinoglycan. Recently, it was demonstrated that *exoB* is required for galactose epimerase activity (7).

The results indicate that infection and some nodule development of plant cells by *Rhizobium* sp. strain NGR234 requires the presence of an acidic EPS molecule, but there is not a strict requirement that the structure of this molecule be identical to that of the wild type. Deletion mutant strain ANU616-d has lost a region of DNA that encodes a cluster of *exo* genes, and consequently the deletion mutant has an Exo^- colony morphology and it produces no detectable level of acidic EPS; however, it produces apparently normal LPS and β-glucan polysaccharides. Strain ANU616-d was unable to infect or invade plant cells, and only callus structures were formed on the roots of *L. leucocephala* plants. This was the same phenotype observed for Tn*5*-generated *exo* mutants of *Rhizobium* sp. strain NGR234 (9). The Exo^- phenotype of ANU616-d could be restored by the introduction of the plasmid pRG100, which carries all the *exo* genes that are specifically required for succinoglycan synthesis in *R. meliloti*. The structure of the EPS synthesized by ANU616-d(pRG100) transconjugants was very similar to that of succinoglycan normally synthesized by *R. meliloti*. Although these transconjugants synthesize a heterologous EPS molecule, they were still capable of invading, infecting, and initiating nodule development on *L. leucocephala* roots, albeit the resulting nodules were Fix^- and smaller than those resulting from symbiosis with the wild-type strain. This is clear evidence that the *Rhizobium* sp. strain NGR234-*L. leucocephala* symbiosis does not require the structure of the acidic EPS to be that of the wild type for the initiation of nodule development, but there is nonetheless a requirement for the presence of an acidic EPS. Comparisons of the structures of EPS molecules generated by *Rhizobium* sp. strain NGR234 and *R. meliloti* show that they have a region of identical carbohydrate structure which involves four glucoses and one galactose, all with the same β linkages. Similar results have been reported by Djordjevic et al. (15) in a study in which either a Sym plasmid-cured strain of *R. leguminosarum* bv. trifolii or EPS purified from *R. leguminosarum* bv. trifolii was used as a coinoculant together with an Exo^- mutant of *Rhizobium* sp. strain NGR234. A combined inoculum could form small Fix^- nodules on *L. leucocephala* (15). The structure of the repeat unit for the acidic EPS of the wild-type *R. leguminosarum* bv. trifolii ANU843 is an octasaccharide with a side chain of four sugars (28). It is interesting to note that the common region of sugars of *Rhizobium* sp. strain NGR234 EPS and *R. meliloti* EPS is also present in the structure of *R. leguminosarum* bv. trifolii EPS, although the β linkages are not the same.

The rhizobia and the host legume form an intimate association which is believed to involve chemical signaling and

recognition between the two partners during the entire course of symbiosis. For the purpose of this discussion, nodule organogenesis can be divided into three stages: *Rhizobium*-host recognition, nodule development with invasion of plant cells, and nitrogen fixation. In the case of *Rhizobium* sp. strain NGR234 and *L. leucocephala*, EPS is not required for the earliest recognition, as evidenced by the appearance of callus structures when *L. leucocephala* seedlings are inoculated with Exo$^-$ mutants such as ANU616-d. This initial recognition step can be expected to involve a lipo-oligosaccharide molecule similar to that described for the *R. meliloti*-alfalfa symbiosis (35). Without the presence of acidic EPS, there is no further development beyond the small disorganized callus growths. The precise role that EPS plays in the continued development of a nodule is unclear. Strain ANU616-d(pRG100) cells synthesize a heterologous EPS molecule and are still able to form organized nodule structures. In contrast, *exoH* mutants of *R. meliloti* lack the succinate group on their acidic EPS molecule and have a greatly decreased LMW EPS fraction (32), but they retain the sugar composition of the wild type and are unable to form bacteroids or invade plant cells (33). Similarly, mutants of *R. meliloti*, designated 101.45 and 688, which produced an excess amount of an acidic EPS (and, in the case of mutant 101.45, may lack the pyruvate group), were unable to invade plant cells or induce infection thread formation (41). The latter two cases are examples of seemingly subtle changes to the EPS structure having dramatic consequences on plant cell invasion. Furthermore, an *expR exoA* mutant or a *mucR exoA* mutant of *R. meliloti* produces only the EPS-II molecule, which is very different in structure to that of succinoglycan, and this mutant is still able to form nitrogen-fixing nodules on alfalfa (21, 55) but not on other plants that are normally hosts for *R. meliloti* (21). Although these mutants have an Exo$^+$ colony morphology, they were as symbiotically ineffective on several other host plants as Exo$^-$ mutants (21). Thus it is apparent that, while the indeterminate nodulating plant does discriminate between structures of acidic EPS molecules synthesized by the invading rhizobia, it is tolerant of certain structural variations. The role of EPS is not passive, but it is also not highly specific for nodule development. This is also evidenced by the fact that in nature, wild-type *R. meliloti* strains can be isolated that have acidic EPS structures different from that of the familiar strain SU47, such as the EPS structure for *R. meliloti* 201 (53). *R. meliloti* 201 produces an acidic EPS molecule that has three different oligosaccharide repeat units containing mannose and GlcA that are not found in *R. meliloti* strain SU47 (1).

The involvement that acidic EPS may play in the transition of an organized Fix$^-$ nodule to one that fixes nitrogen remains unclear. Transposon Tn*5*-generated Exo$^-$ mutants of *Rhizobium* sp. strain NGR234 that are restored to Exo$^+$ by the introduction of pRG100 synthesize the EPS structures of both *Rhizobium* sp. strain NGR234 and *R. meliloti* and also form nitrogen-fixing nodules on *L. leucocephala*. This confirms that it was not the presence of succinoglycanlike EPS that prevented ANU616-d(pRG100) transconjugants from forming nitrogen-fixing nodules; consequently, it may have been the absence of wild-type *Rhizobium* sp. strain NGR234-like EPS. An alternative explanation for the Fix$^-$ phenotype of ANU616-d(pRG100) is that the deletion in strain ANU616-d also resulted in the unavoidable loss of an unidentified gene involved in nitrogen fixation but not in EPS biosynthesis; this would be consistent with the Fix$^-$ phenotype of ANU616-d(R'3222).

The use of deletion strain ANU616-d to construct an *exo* DNA hybrid *Rhizobium* strain proved that the precise structure of the entire acidic EPS molecule of *Rhizobium* sp. strain NGR234 was not essential for the initiation of an organized nodule structure on *L. leucocephala*. Since the *Rhizobium* sp. strain NGR234 *exo* DNA was completely absent from the genome of the ANU616-d(pRG100) transconjugant construct, there was no possibility of low-frequency recombination events, transposon excision events, or suppressor mutations being an explanation for the Nod$^+$ phenotype.

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