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Functional and Evolutionary Relatedness of Genes for Exopolysaccharide Synthesis in *Rhizobium meliloti* and *Rhizobium* sp. Strain NGR234

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***Rhizobium meliloti* SU47 and *Rhizobium* sp. strain NGR234 produce distinct exopolysaccharides that have some similarities in structure. *R. meliloti* has a narrow host range, whereas *Rhizobium* strain NGR234 has a very broad host range. In cross-species complementation and hybridization experiments, we found that several of the genes required for the production of the two polysaccharides were functionally interchangeable and similar in evolutionary origin. NGR234 *exoC* and *exoY* corresponded to *R. meliloti exoB* and *exoF*, respectively. NGR234 *exoD* was found to be an operon that included genes equivalent to *exoM*, *exoA*, and *exoL* in *R. meliloti*. Complementation of *R. meliloti exoP*, *-N*, and *-G* by NGR234 R'3222 indicated that additional equivalent genes remain to be found on the R-prime. We were not able to complement NGR234 *exoB* with *R. meliloti* DNA. In addition to functional and evolutionary equivalence of individual genes, the general organization of the *exo* regions was similar between the two species. It is likely that the same ancestral genes were used in the evolution of both exopolysaccharide biosynthetic pathways and probably of pathways in other species as well.**

Many bacteria secrete exopolysaccharide (EPS), which may remain attached to the cell surface as a capsule or may be released into the surrounding medium (30). Much remains to be learned concerning the genetics and biochemistry of bacterial EPS production, especially with regard to the biochemical functions of individual gene products. Within the genus *Rhizobium*, numerous mutants in EPS production have been isolated (3, 8–10, 17, 18, 20, 21), but their characterization with regard to the bacterium-plant interaction has tended to take precedence over biochemical studies. For both *Rhizobium* sp. strain NGR234 (8) and *Rhizobium meliloti* SU47 (21), EPS has been shown to be necessary for normal nodulation. In addition, EPS may be involved in host range. *Rhizobium* sp. strain NGR234 probably has the broadest host range of any *Rhizobium* or *Bradyrhizobium* as it can infect and nodulate a large range of tropical legumes and the nonlegume *Parasponia andersonii* (32). In contrast, *R. meliloti* strain SU47 is a narrow-host-range *Rhizobium* which nodulates the alfalfa cross-inoculation group consisting only of *Medicago*, *Melilotus*, and *Trigonella*. Evidence implicating the involvement of EPS in host range was seen when cloned *R. trifolii hsn* (host-specific nodulation) genes were transferred into *R. leguminosarum* biovar *viciae* (26). The hybrids synthesized *R. trifolii* type-acidic EPS and were able to nodulate white clover efficiently. Similarly, when the *Rhizobium* sp. strain NGR234 Sym (symbiotic) plasmid was mobilized into *R. meliloti*, the host range was extended to the tropical legume siratro (a host for *Rhizobium* sp. strain NGR234 [24]). However, the structures of the EPS of these hybrids was not examined.

An understanding of the biosynthetic defects of *Rhizobium* EPS mutants would both advance the study of polysaccharide biosynthesis and lend credence to our interpretations of the symbiotic effects. In this report, while we do not offer a biochemical characterization, we do use genetic methods to show functional equivalence between EPS genes of *R. meliloti* SU47 and *Rhizobium* sp. strain NGR234. We also find, by cross-species hybridization analysis, that many functionally equivalent EPS genes are homologous. Since the two polysaccharides have certain structural similarities, and are necessary for nodule invasion in both cases, the impact of future biochemical and regulatory studies in either species may be extendable to the other.

The EPS produced by *R. meliloti* SU47 is succinoglycan, a polymer of octasaccharide subunits that contain seven glucose units and one galactose, all in various β linkages, as well as approximately one pyruvate, one acetate, and one succinate group, per subunit (1; Fig. 1B). In comparison, the *Rhizobium* sp. strain NGR234 EPS has a nonasaccharide repeat unit that contains five glucoses, two galactoses, and two glucuronic acids, all in various α and β linkages, and one pyruvate and one acetate group (11; Fig. 1A). The two oligosaccharide repeat units, although different, have a common region of four glucoses and one galactose, all with the same β linkages (boxed regions of Fig. 1). Succinoglycan can be stained by the fluorescent dye Calcofluor (19, 21), while the NGR234 EPS cannot (this study). Tolmasky et al. (31) have shown that the succinoglycan subunit is assembled as a polyprenyl glycosyl diphosphate, beginning with the addition of the galactosyl unit to the lipid carrier.

exo genes, required for EPS synthesis, have been identified in both species. In *R. meliloti*, a cluster of genes on the megaplasmid pRmeSU47b (14) contains seven known complementation groups (*exoP*, *-M*, *-A*, *-L*, *-F*, *-Q*, and *-B*; Fig. 2) in which mutants absolutely abolish succinoglycan synthesis (23). In addition, the cluster contains three complementation groups (*exoN*, *-J*, and *-G*) in which mutants appear to produce a decreased quantity of succinoglycan which is altered in distribution between high- and low-molecular-weight fractions (23). Mutants in two groups in the *exo* cluster (*exoK* and *exoH*) are deficient in the forma-

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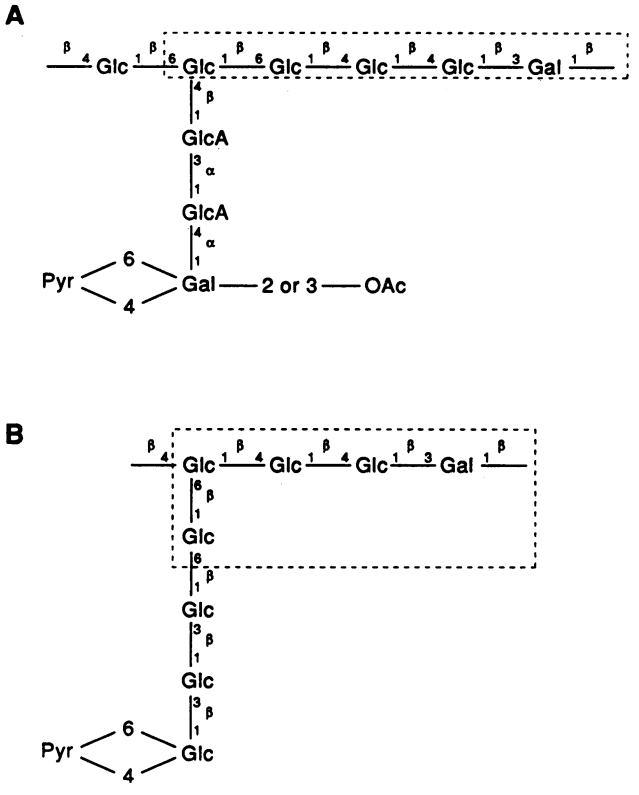

FIG. 1. Structures of the (A) NGR234 EPS (11) and the (B) *R. meliloti* EPS, succinoglycan (1). In succinoglycan, O-succinyl and O-acetyl groups are present at undetermined sites. Note that, starting from the right, the first five sugars and linkages in the two polysaccharides are identical (boxed).

tion of a characteristic halo of fluorescence around the colonies on Calcofluor agar. *exoK* mutants are delayed in halo formation, while *exoH* mutants lack any halo. *exoH* mutants produce nonsuccinylated succinoglycan (20) and lack a low-molecular-weight fraction of succinoglycan usually produced by the wild-type strain (19). Group E mutants are deletions of major portions of the *exo* region (13). *exoC*, which maps to the chromosome rather than the megaplasmid (14), is necessary for phosphoglucomutase activity (33) and is required for production of the periplasmic cyclic glucan and the O chain of the lipopolysaccharide as well as for succinoglycan production (19). *exoD*, which also maps to the chromosome (14), appears to modulate the quantity of succinoglycan and its distribution between high- and low-molecular-weight fractions (19).

In NGR234, the *exo* gene cluster (9) contains four complementation groups, *exoB* (formerly *A-B*), *-D*, *-Y* (formerly *E-F*), and *-C* (Fig. 2). An additional complementation group, *exoG*, does not map to the cluster. Mutations in any of these groups abolish EPS production. Recent evidence suggests that *exoY*, together with a linked gene, *exoX*, is a regulator of EPS production; *exoX* may be analogous to the *R. leguminosarum psi* (polysaccharide inhibition) gene (16).

It is of great interest to define the *exo* genes that are common to the two species (i.e., function interchangebly in the synthesis of both polysaccharides) and those that are specific. This will help us eventually to understand the functions of the *exo* genes in the biosynthesis of EPS. In this paper, we report that most *R. meliloti exo* mutants can be complemented by R'3222, which carries NGR234 wild-type *exo* DNA. Conversely, three groups of NGR234 *exo* mutants can be complemented by *R. meliloti exo*-complementing cosmids. The general organization of the *exo* genes is similar between the two species.

MATERIALS AND METHODS

Strains, plasmids, and media. Bacterial strains, plasmids, and transducing phage are listed in Table 1. The NGR234 strain, ANU2820, has been designated *exoA* (9). However, this strain behaves as though it is a double mutant containing Tn*5* in the *exoB* locus and a second, perhaps spontaneous mutation in *exoC*. It contains only one Tn*5* insertion, in the same site as the *exoB* mutant ANU2826 (J. Gray, unpublished data). The failure of the *exoA* mutant recombinant of R3222, R'2820 (9), to complement NGR234 *exoB* and *exoC* mutants suggests that ANU2820 is an *exoB exoC* double mutant. *R. meliloti* strains were grown on LB medium or yeast-mannitol (YM) medium as described before (35). For liquid culture, LB medium was supplemented with $CaCl_2$ and $MgSO_4$, each at 2.5 mM final concentration. For NGR234 strains, YM was used. Concentrations of antibiotics were as described previously (35). Calcofluor white M2R (Cellufluor) was obtained from Polysciences, Warrington, Pa., or Sigma Chemical Co., St. Louis, Mo., and was added to agar at a final concentration of 0.02%.

Genetic techniques and cloning. Cosmids were transferred conjugatively in triparental matings as described before (35), and R-primes were transferred in biparental matings (9). Tn*5* insertion mutagenesis of cosmids, recombination of insertions into genomic DNA by homogenitization, transduction of Tn*5* markers, and DNA techniques were as described before (35). pSP329 (a gift from Sandy Porter [unpublished data]) was constructed by cloning the 0.4-kilobase (kb) *Hae*II fragment which contains the polylinker and the α-complementation-specifying region from pUC18 into the broad-host-range, mobilizable plasmid pTJS75 (28) which had been partially digested with *Hae*II. pHZ401 was constructed by cloning the 2.5-kb *Hin*dIII-*Eco*RI fragment of pEX80 into pSP329. pHZ400 and pHZ405 are pSP329 containing the 4.8-kb *Eco*RI-*Hin*dIII fragment from pEX80 and the 2.9-kb *Hin*dIII-*Bgl*II fragment from pEX154, respectively.

Nodulation assays. Growth and inoculation of *Medicago sativa* (alfalfa) cv. Iroquois and assays for nitrogen fixation were performed by acetylene reduction as described previously (35). Nitrogen fixation was measured 4 weeks after inoculation. Growth and inoculation of *Leucaena leucocephala* var. Peru was as described previously (9).

Isolation and analysis of polysaccharide. Calcofluor staining of colonies was performed as described before (21). EPS was produced in liquid culture by growing for 5 days in a salts-mannitol-glutamate medium as described previously (19). EPS was prepared from concentrated culture supernatant either by dialysis or by fractionation into high- and low-molecular-weight forms by Bio-Gel A5m chromatography followed by desalting, as described previously (19). Quantitation of EPS by the anthrone-sulfuric acid method and proton-nuclear magnetic resonance (NMR) spectroscopy were as described previously (19).

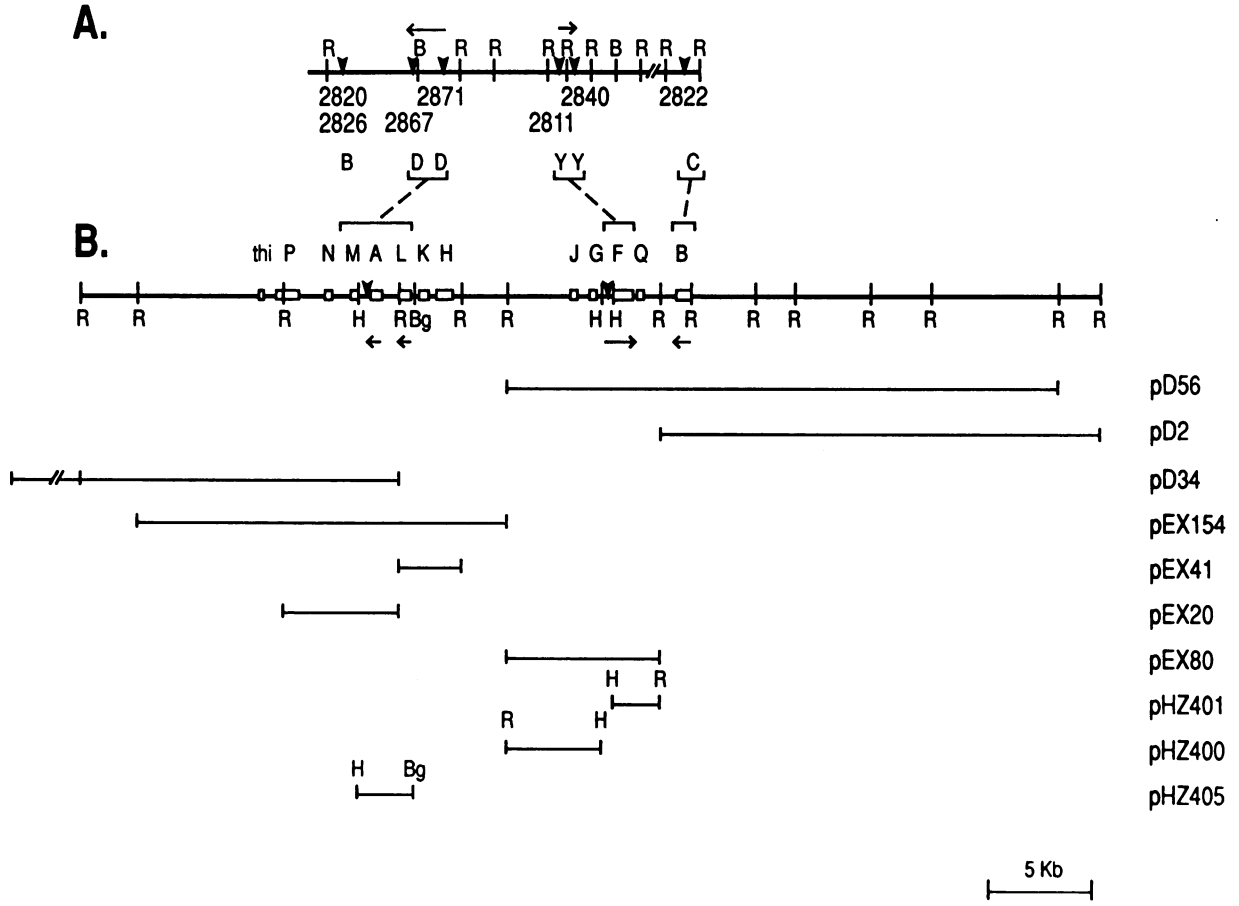

FIG. 2. Partial restriction map of the *exo* gene cluster of NGR234, modified from Chen et al. (9) (A) and map of the *R. meliloti exo* gene cluster and corresponding plasmid inserts (modified from Long et al. [23]) (B). *exo* gene designations are shown below the line in part A and above the line in part B. A thiamine biosynthetic gene (*thi* [14]) is also shown in part B. The correspondence between NGR234 and *R. meliloti exo* genes is shown with broken lines and brackets. Arrowheads indicate Tn*5* insertion sites of the NGR234 mutants indicated and (left to right) of *R. meliloti* mutant plasmid pEX154-ANU280*exoD*::Tn*5* (Table 5) and strain Rm7210 (Table 4). Horizontal arrows indicate direction of transcription. Restriction sites are indicated as follows: R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; Bg, *Bgl*II.

RESULTS

Complementation of *R. meliloti exo* mutants with NGR234 R'3222. To test the ability of NGR234 *exo* genes to function in place of *R. meliloti exo* genes, we introduced the NGR234 R-prime, R'3222 (Fig. 2), into various *R. meliloti* mutants and observed fluorescence of colonies on Calcofluor agar (Table 2). *R. meliloti* mutants, *exoA*, *-B*, *-F*, *-L*, *-M*, and *-P* (see Table 1 for strain descriptions), all completely deficient in succinoglycan production, recovered Calcofluor staining in the presence of R'3222. To confirm that Calcofluor staining correlated with the production of succinoglycan, we harvested EPS produced by *R. meliloti exoF* (R'3222) and *exoP* (R'3222) in liquid culture. The quantities produced were almost the same as with the wild-type parental strain Rm1021, and the proton-NMR spectra were similar to those of authentic succinoglycan (see Fig. 3 for *exoF* [R'3222]). The Calcofluor staining together with the NMR data and the fact that the EPS is produced in the *R. meliloti* background make it highly probable that the EPS being produced in the complemented strains is succinoglycan. *exoG* and *exoN*, with partial defects, increased in Calcofluor fluorescence with R'3222.

In contrast, *exoC* (completely defective in succinoglycan production), *exoD* and *exoJ* (partially defective), and *exoK* and *exoH* (delayed halo and haloless mutants) showed no change in Calcofluor staining when containing R'3222. A proton-NMR spectrum of EPS from *exoH* (R'3222) revealed nonsuccinylated succinglycan, as is produced by *exoH* itself. For the chromosomal mutants, *exoC* and *exoD*, the lack of complementation is not surprising, since linkage of any counterpart genes in NGR234 to the *exo* cluster would not be expected. Nor did we expect complementation of the nonsuccinylating *exoH* mutant, since the NGR234 EPS does not contain succinate; this gene, then, is likely specific to succinoglycan synthesis. For *exoJ* and *exoK*, the results could likewise indicate that no counterpart exists for NGR234 EPS synthesis or that such a counterpart gene is not contained on R'3222 or is not expressed in *R. meliloti*.

Complementation of the *exoQ* mutant with R'3222 yielded variable results. Most colonies were Calcofluor nonfluorescent, but some were fluorescent. Fluorescent colonies often gave rise to nonfluorescent or partially fluorescent derivatives. We have not examined this further.

We tested the relationship between restored Calcofluor staining and nodulation effectiveness. In most cases in which the succinoglycan phenotype was restored by complementation, the Fix phenotype, as determined by plant growth and acetylene reduction, was comparable to the wild-type

TABLE 1. Bacterial strains, plasmids, and transducing phage

Strain or plasmid	Relevant properties	Source or reference
R. meliloti		
SU47	Wild type	34
Rm1021	SU47 *str-21*	F. Ausubel
Rm5000	SU47 *rif-5*	T. Finan
Rm7011	Rm5000 *exoA11*::Tn*5*	21
Rm7031	Rm1021 *exoA31*::Tn*5*	21
Rm7013	Rm5000 *exoB13*::Tn*5*	21
Rm7015	Rm5000 *exoC15*::Tn*5*	21
Rm7017	Rm1021 *exoD17*::Tn*5*	21
Rm7022	Rm1021Ω7022::Tn*5*, Group E Exo$^-$	21
Rm7029	Rm1021Ω7029::Tn*5*, group E Exo$^-$	21
Rm7055	Rm1021 *exoF55*::Tn*5*	21
Rm7210	Rm1021 *exoF210*::Tn*5*	This work
Rm8302	Rm1021 *exoG302*::Tn*5*	23
Rm7154	Rm1021 *exoH154*::Tn*5*	20
Rm8319	Rm1021 *exoJ319*::Tn*5*	23
Rm8476	Rm1021 *exoK476*::Tn*5*	23
Rm8431	Rm1021 *exoL431*::Tn*5*	23
Rm8457	Rm1021 *exoM457*::Tn*5*	23
Rm8416	Rm1021 *exoN416*::Tn*5*	23
Rm8468	Rm1021 *exoP468*::Tn*5*	23
Rm8332	Rm1021 *exoQ332*::Tn*5*	23
Rhizobium sp. strain NGR234		
NGR234	Wild type, broad-host-range cowpea *Rhizobium*	9
ANU280	Sm^r Rif^r derivative of NGR234	9
ANU2820	ANU280 *exoA20*::Tn*5* (*exoB-C*)	9
ANU2826	ANU280 *exoB26*::Tn*5*	9
ANU2822	ANU280 *exoC22*::Tn*5*	9
ANU2871	ANU280 *exoD71*::Tn*5*	9
ANU2840	ANU280 *exoY40*::Tn*5*	9
ANU2811	ANU280 *exoY11*::Tn*5*	9
E. coli		
MM294A	*pro-82 thi-1 endA1 hsdR17 supE44*	G. Walker
MT614	MM294A *recA56*::Tn*5*	T. Finan
MT609	p3473 *polA1 thy* Spc^r	T. Finan
HB101	*rpsL20 proA2 recA13 hsdS20* (r_B^- m_B^-) *supE44*	G. Walker
C2110	*polA1 rha his*	E. Nester
DH5α	Transformation recipient	S. Lory
Plasmid		
pRK2013	Nm^r, ColE1 replicon with RK2 *tra* genes	G. Ditta
pRK600	Cm^r Nm^s, pRK2013 Nm::Tn*9*	14
pLAFR1 clone bank	Tc^r, *R. meliloti* genes on cosmid pLAFR1	F. Ausubel
pD2	pLAFR1 *exoB*-complementing plasmid	21
pD34	pLAFR1 *exoA*-complementing plasmid	21
pD56	pLAFR1 *exoBF*-complementing plasmid	21
pEX154	pLAFR1 *exoAH*-complementing plasmid	20
pEX154-*exoA407*::Tn*5*	Tn*5* insertion in *exoA*	23
pEX154-*exoM457*::Tn*5*	Tn*5* insertion in *exoM*	23
pSUP104	Cm^r Tc^r IncQ	29
pEX41	pSUP104 *exoH*-complementing subclone	23
pEX20	pSUP104 *exoA*-complementing subclone	23
pEX80	pSUP104 *exoF*-complementing subclone	23
pPH1JI	IncP Gm^r Sp^r *tra*$^+$	F. Ausubel
pSP329	Broad-host-range vector, Tc^r (see Materials and Methods)	S. Porter
pHZ400	4.8-kb *Hin*dIII-*Eco*RI fragment cloned into pSP329	This work
pHZ401	2.5-kb *Hin*dIII-*Eco*RI fragment cloned into pSP329	This work
pHZ405	2.9-kb *Bgl*II-*Hin*dIII fragment cloned into pSP329	This work
R'3222	R68.45 R-prime carrying wild-type NGR234 *exo* genes	9
R'2822	R'3222 *exoC22*::Tn*5*	9
R'2867	R'3222 *exoD67*::Tn*5*	9
R'2871	R'3222 *exoD71*::Tn*5*	9
R'2811	R'3222 *exoY11*::Tn*5*	9
R'2840	R'3222 *exoY40*::Tn*5*	9
Phage		
ϕM12	Generalized transducing phage for *R. meliloti SU47*	T. Finan

TABLE 2. Complementation of *R. meliloti exo* mutants by R'3222

Strain[a]	Calcofluor fluorescence[b]	Fix[c]
Rm1021 (wild type)	+	+
exoA, *-B*, *-F*, *-L*, *-M*, *-P*	−	−
R'3222 transconjugates of *exoA*, *-B*, *-F*, *-M*, *-P*	+	+
exoC, group E	−	−
R'3222 transconjugates of *exoC*, group E	−	−
exoQ	−	−
R'3222 transconjugants of *exoQ*	Variable	−
exoG	+/−	+/−
exoG (R'3222)	+	+/−[d]
exoN	+/−	+
exoN (R'3222)	+	+
exoD	+ −	−
exoD (R'3222)	+/−	−
exoJ	+/−	+/−
exoJ (R'3222)	+/−	+/−
exoK	+[e]	+
exoK (R'3222)	+[e]	+
exoH	+[f]	−
exoH (R'3222)	+[f]	−

[a] See Table 1 for description of strains. The *exoA* strain was Rm7031. Both group E mutants were tested.
[b] Fluorescence was determined on LB agar containing calcofluor: +, fluorescent; −, nonflorescent; +/−, intermediate fluorescence.
[c] Nitrogen fixation determined by plant growth and acetylene reduction. For each strain, at least six plants were tested. +, Nitrogen fixation equivalent to wild type, nodules cylindrical and pink; −, no detectable nitrogen fixation, nodules round and white; +/−, intermediate level of fixation, nodules of both types present.
[d] Nitrogen fixation intermediate between + and −, but strains containing R'3222 induced a higher proportion of cylindrical, pink, acetylene-reducing nodules on alfalfa plants than strains without R'3222.
[e] Delayed halo formation.
[f] No halo, succinoglycan nonsuccinylated.

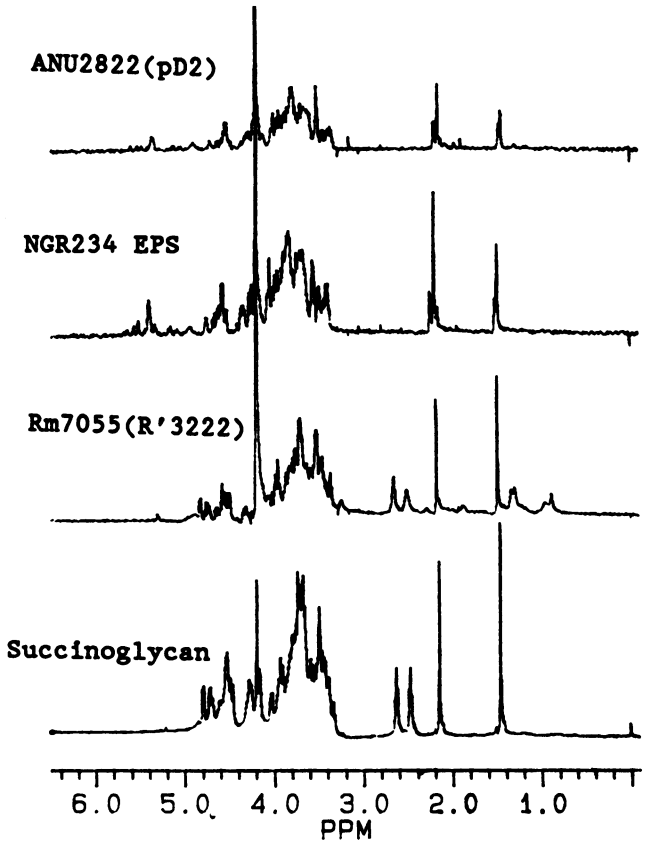

FIG. 3. Proton-NMR spectra. Peaks corresponding to pyruvate and acetate are at approximately 1.5 and 2.2 ppm, respectively. Succinate peaks (present only in succinoglycan) are at approximately 2.5 and 2.65 ppm. The complex regions from 3.3 to 4.9 ppm represent protons of the carbohydrate constituents. The NGR234 EPS [obtained from ANU2820(R'3222)] has stoichiometric amounts of characteristic α-anomeric protons that resonate at approximately 5.4 ppm, while succinoglycan (obtained from Rm1021) has only a small peak at 5.2 ppm due to anomerization of the terminal sugar of the oligosaccharide form. In the spectrum of Rm7055(R'3222), the peaks at 0.8 and 1.3 ppm appear to be contaminants. The solvent peak (HDO) is at 4.2 ppm.

strain Rm1021 (Table 2). The only exception was with *exoG* (R'3222), which induced the formation of small, white, bacteroid-free nodules as well as normal nodules. Even here, though, the proportion of normal nodules was greater with *exoG* (R'3222) than with *exoG*.

Complementation of NGR234 *exo* mutants with *R. meliloti exo* cosmids. *Rhizobium* sp. strain NGR234 and its streptomycin-resistant, rifampin-resistant derivative ANU280 produce mucoid colonies on YM agar, while *exo* mutant derivatives form dry colonies. Using mucoid colony appearance as an indication of EPS production, we confirmed that the NGR234 R-prime, R'3222, complements the NGR234 mutants *exoB*, *-C*, *-D*, and *-Y*, as well as ANU2820 (formerly designated *exoA*, but apparently an *exoB exoC* double mutant; see Materials and Methods). In cross-species complementation experiments, *R. meliloti* cosmids complemented NGR234 mutants as shown (Table 3). To confirm that the complemented mutants produced the NGR234 EPS, we showed that the proton-NMR spectra of EPS from ANU2822(pD2), ANU2822(R'3222), and the parental strain ANU280 were the same (Fig. 3). Furthermore, the colonies were Calcofluor nonfluorescent. The quantities of EPS produced by ANU2822(pD2), ANU2822(pD56), ANU2871(pEX154), ANU2811(pD56), and ANU280 were similar. The EPS produced by ANU2822(pD2) and ANU2822(pD56) was sufficient for nodule invasion of *Leucaena*, since plants inoculated with these constructs fixed nitrogen. These complementation results indicated that a functional counterpart of NGR234 *exoD* lay in the *R. meliloti exoP-N-M-A-L-K-H*

TABLE 3. Complementation of NGR234 *exo* mutants[a]

Strain	Mutant class	Plasmid					
		R'3222	pD34	pEX154	pD56	pD2	pD15
ANU2871	*exoD*	+	−	+	−	−	−
ANU2811	*exoY*	+	−	−	+	−	−
ANU2840	*exoY*	+	−	−	+/−	−	−
ANU2822	*exoC*	+	−	−	+	+	−
ANU2826	*exoB*	+	−	−	−	−	−
ANU2820	A[b]	+	−	−	−	−	−

[a] Colonies were grown on YM agar with Calcofluor. +, Mucoid colonies; −, dry colonies. All colonies were nonfluorescent.
[b] ANU2820 is probably an *exoB exoC* double mutant (see text).

region; of NGR234 *exoY*, in the *R. meliloti exoJ-G-F-Q* region; and of NGR234 *exoC*, in the *R. meliloti exoB* region (Fig. 2). We were unable to complement NGR234 *exoB* with any known *R. meliloti* cosmids or with the *R. meliloti* clone bank. Of the two NGR234 *exoY* mutants tested, only one (ANU2811) was fully complemented. The other mutant (ANU2840), when containing pD56, developed mucoidy after prolonged incubation. (ANU2840 without pD56 also became mucoid, but less so than with pD56.) It has been shown that both of these mutations arose from insertions in the same open reading frame (16). One explanation for their difference in complementability is that ANU2840, bearing the more downstream insertion, expresses a truncated protein that interferes with the activity of the *R. meliloti* counterpart protein. Alternatively, ANU2840 could have a second mutation in another locus. In conclusion, NGR234 *exoC*, *-D*, and *-Y* mutants were complemented by *R. meliloti* DNA from the *exo* region. These results indicated the rough locations of the counterpart genes in *R. meliloti* and led to the finer determinations described below.

***R. meliloti exoB* gene equivalent to NGR234 *exoC* gene.** The ability of the *R. meliloti* cosmid pD2 (as well as pD56) to complement the NGR234 *exoC* mutant suggested that *R. meliloti exoB* might be the corresponding gene (Fig. 2). Indeed, pD56*exoB347*::Tn*5* did not complement ANU280 *exoC*. As a control, we showed that this mutant cosmid did complement ANU2811 (ANU280 *exoY*). Furthermore, R'2822, which failed to complement ANU280 *exoC*, also failed to complement *R. meliloti exoB*. Thus, the two genes from the two species are functionally interchangeable. They were apparently similar at the DNA sequence level, too. We obtained *Eco*RI-digested genomic DNA of ANU280 and ANU280 *exoC*, as well as *Eco*RI-digested DNA of R'3222 and R'2822 (R'3222*exoC*::Tn*5*) and probed with a 1.4-kb *Eco*RI fragment containing most of the *R. meliloti* exoB gene (Fig. 2). A strong band in the digests of ANU280 and R'3222 increased by 5.7 kb in ANU280 *exoC* and R'2822 (not shown). These results show that the DNA spanning the NGR234 *exoC* gene is similar to DNA on the *R. meliloti* *Eco*RI fragment containing *exoB*.

***R. meliloti exoF* gene equivalent to NGR234 *exoY* gene.** Since pD56 but not pD2 complemented ANU280 *exoY*, it was likely that the corresponding gene in *R. meliloti* was on the 8-kb *Eco*RI fragment (Fig. 2). Indeed, pEX80 (Fig. 2) restored EPS production to the NGR234 *exoY* mutant, ANU2811 (Table 4). We found that the mutant *R. meliloti* cosmid, pD56*exoF306*::Tn*5* (23), only partially complemented *R. meliloti exoF55*::Tn*5* (Table 4). As expected if *R. meliloti exoF* and NGR234 *exoY* correspond, the same cosmid also had a partial effect on ANU2811. In another test of this correspondence, we mutagenized pD56 with Tn*5*, mobilized it en masse into ANU2811, and screened for dry colonies. We were able to isolate a mutant cosmid, designated pD56*exoF210*::Tn*5*, that consistently failed to complement both ANU2811 and *R. meliloti exoF55* (Table 4). We transferred the new mutation into the Rm1021 genome by homogenitization and designated the new strain Rm7210. As expected, Rm7210 was nonfluorescent on Calcofluor agar and was only partially complemented by pD56*exoF306*::Tn*5*.

Fortuitously, our results enable us to extend the 5' minimum map boundary of the *R. meliloti exoF* gene. The Tn*5* insertion site of Rm7210 turned out to be within a 0.75-kb *Hin*dIII fragment that lies between the minimum boundaries of *exoG* and *exoF* as designated previously (Fig. 2). Plasmid pHZ401, which carries a 2.5-kb *Hin*dIII-*Eco*RI fragment (Fig. 2), complemented *exoQ*, but failed to complement *exoF* (Table 4). Taken together, these observations indicate that the minimum 5' boundary of the *exoF* gene should be extended to the *Hin*dIII site of pHZ401. Müller et al. (25) also reported an *R. meliloti* SU47 *exo* mutant with Tn*5* inserted in the 0.75-kb *Hin*dIII fragment. The *exoF* region corresponds to region III of Keller et al. (17).

TABLE 4. Complementation of *R. meliloti exoF* and *exoQ* and NGR234 *exoY* mutants by various recombinant plasmids[a]

Plasmid	Strain and mutation			
	R. meliloti			NGR234 [ANU2811 (*exoY11*)]
	Rm7055 (*exoF55*)	Rm7210 (*exoF210*)	Rm8332 (*exoQ332*)	
pD56	+	+	+	+
pD2	−	−	−	−
pEX80	+	+	+	+
pD56*exoF306*::Tn*5*	+/−	+/−	+	+/−
pD56*exoF210*::Tn*5*	−	−	ND	−
pD56*exoQ332*::Tn*5*	+	+	−	+
pHZ401	−	−	+	−
pHZ400	−	−	−	−

[a] For *R. meliloti* strains, +, −, and +/− refer to degree of colony fluorescence on LB agar with Calcofluor. For NGR234 strains, +, −, and +/− refer to degree of colony mucoidy on YM agar. ND, Not determined.

In agreement with our results, Gray et al. (16) reported previously that an internal NGR234 *exoY* probe hybridized strongly to digests of DNA contained in the *R. meliloti* cosmid pD56, but did not appear to flank Tn*5* insertions in the region. The region of homology was pinpointed by the fact that the hybridizing bands included the 0.75-kb *Hin*dIII fragment.

The NGR234 *exoD* locus includes genes equivalent to *R. meliloti exoL*, *exoA*, and *exoM*. The complementation of ANU280 *exoD* with pEX154 but not with pD34 (Table 2) was surprising, since according to the published map (23) pD34 contains all DNA present in pEX154. Even after 10 days of incubation on YM agar plates, colonies of ANU280 *exoD* (pD34) were dry. In liquid medium, ANU280 *exoD* (pD34) produced essentially no EPS. We were unable to interpret this result until we remapped pD34 (Fig. 2). We found that pD34 lacks the 3.6-kb *Eco*RI fragment containing *exoL*, *-K*, and *-H* and its adjacent 2.3-kb *Eco*RI fragment. Consistent with this, pD34 complemented *R. meliloti exoA* but not *exoL*.

Our complementation results are summarized in Table 5. The failure of NGR234 R'2871 (R'3222*exoD71*::Tn*5*) to complement *R. meliloti exoA*, *-L*, and *-M* indicated that the NGR234 *exoD* gene is an operon that includes the respective *R. meliloti* equivalent genes. Consistent with this, the *R. meliloti* plasmids pD34, pEX20, pEX41, and pHZ405 (Fig. 2) all failed to complement ANU280 *exoD71*. R'2867 (R'3222*exoD67*::Tn*5*) failed to complement only *R. meliloti exoA*, indicating that transcription of NGR234 *exoD* is from right to left and that the equivalent of *R. meliloti exoA* is toward the 3' end.

In an effort to find further evidence for *R. meliloti* genes with equivalents in NGR234 *exoD*, we pooled random Tn*5* insertions in pEX154 and mobilized them en masse into ANU2871 (*exoD71*). We were able to isolate the cosmid pEX154-ANU280*exoD*::Tn*5*, which consistently failed to complement ANU2871. This cosmid failed to complement Rm7011 (*exoA11*::Tn*5*), but complemented Rm7031 (*exoA31*::Tn*5*). The simplest explanation for the differing results with respect to *exoA31* and *exoA11* would be that the

TABLE 5. Complementation of NGR234 *exoD* and corresponding *R. meliloti* mutants by various plasmids[a]

Plasmid	Strain and mutation						
	R. meliloti						NGR234 [ANU2871 (*exoD71*)]
	Rm7031 (*exoA*)	Rm8431 (*exoL*)	Rm8457 (*exoM*)	Rm8416 (*exoN*)	Rm8468 (*exoP*)	Rm7011 (*exoA*)	
R'3222	+	+	+	+	+	+	+
R'2871 (*exoD781*)	−	−	−	+	+	−	−
R'2867 (*exoD67*)	−	+	+	+	+	−	−
pD34	+	−	+	ND[b]	ND	+	−
pEX20	+	−	ND	ND	ND	ND	−
pEX41	−	−	ND	ND	ND	ND	−
pHZ405	+	+	−	+/−	−	+	−
pEX154	+	+	+	ND	+	+	+
pEX154-ANU280-*exoD*::Tn*5*	+	+	+	+	+	−	−
pEX154-*exoA407*::Tn*5*	−	ND	+	ND	ND	−	ND
pEX154-*exoM457*::Tn*5*	+	ND	−	ND	ND	+	ND

[a] For *R. meliloti* strains, +, −, and +/− refer to degree of colony fluorescence on LB agar with Calcofluor. For NGR234 strains, + and − refer to degree of colony mucoidy on YM agar.
[b] ND, Not determined.

two mutations actually belong to separate complementation groups, *exoA11* corresponding to the new insertion in pEX154. However, both *exoA11* and *exoA31* appeared to belong to the same complementation group (Table 5). We can offer no obvious explanation for these results. The insertion site of pEX154-ANU280*exoD*::Tn*5* mapped 1.6 kb to the left from the *Eco*RI site that bounds the pD34 insert, at the 5′ end of *exoA* (Fig. 2). Homogenotes were nonfluorescent with Calcofluor. The insertion site of Rm7011 has not been mapped.

Data from cross-species hybridization were consistent with the results of complementation experiments. Beginning at the left portion of NGR234 *exoD* (Fig. 2), we found that a 9-kb *Bam*HI fragment containing the site of insertion of *exoD67*::Tn*5* hybridized strongly to the 5.8-kb *Eco*RI fragment of pD34 that contains *exoA* and *exoM*. Moving to the right, the 2.2-kb *Bam*HI-*Eco*RI fragment containing the upstream portion of NGR234 *exoD* showed strong hybridization only to the 3.3-kb *Eco*RI fragment of *R. meliloti* genomic DNA that contains *exoL*. Furthermore, we used the 0.9-kb *Eco*RI-*Bgl*II fragment that contains *R. meliloti exoL* as a probe against *Eco*RI-*Bam*HI double-digested DNA from ANU280, ANU2871, R'2867 (R'3222*exoD67*::Tn*5*), and R'2871 (R'3222*exoD71*::Tn*5*). We found only a strong 2.2-kb band in ANU280 and R'2867, but two bands of 3.5 and 4.4 kb in ANU2871 and R'2871. These results indicate that the insertion site of ANU280*exoD71* is in the counterpart of the *R. meliloti exoL* gene. Taken together with the complementation results, the hybridization data indicate that, of the genes included in NGR234 *exoD*, *exoL* and its counterpart are rightmost in both species, but the *exoA* counterpart is leftmost only in NGR234.

The results of complementation with pHZ405 add some information to the *R. meliloti* map. The entire *exoA* and *exoL* genes, but not *exoM*, lie between the *Hin*dIII and *Bgl*II sites (Fig. 2).

DISCUSSION

The two *Rhizobium* species, *R. meliloti* SU47 and *Rhizobium* sp. strain NGR234, have very different host range capabilities and produce EPS that differ significantly in structure. In this paper, we have shown that many of the genes required for the production of each species' respective EPS are functionally interchangeable. Thus, from genetic complementation analysis, we showed that NGR234 *exoY* was equivalent to *R. meliloti exoF*, NGR234 *exoC* was equivalent to *R. meliloti exoB*, and NGR234 *exoD*, apparently a polycistronic operon, was equivalent to *R. meliloti exoM*, *-A*, and *-L*. In addition, the ability of the NGR234 R-prime, R'3222, to complement the *R. meliloti exoP*, *-G*, and *-N* mutants suggests that additional equivalent genes are still to be identified on R'3222. On the other hand, genes that are specific for one EPS or the other must exist. It is possible that the NGR234 *exoB* is specific for NGR234 EPS synthesis, since we were unable to find an *R. meliloti* counterpart by complementation with the *R. meliloti* clone bank.

The extensive similarity in gene function summarized above can be explained in several ways. In the case of NGR234 *exoY*-*R. meliloti exoF*, the genes probably play similar regulatory roles. Gray et al. (16) have reported that the Exo phenotypes of certain NGR234 derivatives depend on the relative gene dosages of *exoY* and another gene, *exoX*. The phenotypic effect and predicted protein structure of *exoX* are reminiscent of the *R. leguminosarum psi* gene (4–6).

The pleiotropic effects of *R. meliloti exoB* mutants suggest that this gene and the NGR234 *exoC* gene may function similarly in biosynthetic steps not specific to any particular exopolysaccharide. *R. meliloti exoB* mutants are defective in the synthesis of three different polysaccharides: succinoglycan, the second *R. meliloti* EPS, EPSb (35) or EPSII (15), and normal lipopolysaccharide (19). These genes, then, could function in the synthesis of common precursors or in a nonspecific secretion mechanism.

For the remaining genes common to the two species, two possibilities exist. First, postpolymerizational modification (if any) and secretion mechanisms could be similar for the two polysaccharides. Roberts et al. (27) and Boulnois and Jann (7) reported that the gene clusters necessary for these functions in *Escherichia coli* strains that produce different group II capsular polysaccharides were organized similarly and were functionally interchangeable.

The second explanation lies in the common structural features of NGR234 and *R. meliloti* EPS. Starting with the galactose, shown by Tolmaski et al. (31) to be the first residue in the assembly of the oligosaccharide subunit of succinoglycan, the first five additions to the chain are identical in composition and linkage in the two polysaccha-

rides (Fig. 1). It seems likely, then, that NGR234 *exoD* and the corresponding *R. meliloti* genes *exoM*, *-A*, and *-L*, as well as other common genes that may exist, represent the evolution of an oligosaccharide assembly pathway that remains common to both species.

The common evolution of the *exo* genes that are functionally equivalent in both species is indicated by DNA sequence similarity and gene organization as well as functional interchangeability. In every case where cross-species complementation occurred, we were able to detect strong hybridization of DNA fragments at or near the Tn*5* insertion sites of the mutants. Also, the overall order of the equivalent genes was similar in the two species; NGR234 *exoD*, *-Y*, and *-C* corresponded to *R. meliloti exoM-A-L*, *-F*, and *-B*, respectively (Fig. 2). Directions of transcription, when known, were also similar; the *R. meliloti exoA* and *exoL* genes were found to be transcribed from right to left as is the NGR234 *exoD* operon, and *R. meliloti exoF* (23) is transcribed from left to right as is NGR234 *exoY* (16). The only case we found in which gene order differed was when *R. meliloti exoM* and *exoA* were reversed with respect to the corresponding genes within the NGR234 *exoD* operon. It is worth noting that EPS of certain (but by no means all) strains of *R. trifolii* and *R. lupini* (2) also have structures that begin with the same five residues and linkages that are common between the *R. meliloti* SU47 and NGR234 EPS. It is likely that the same ancestral genes were used repeatedly in the evolution of various EPS biosynthetic pathways.

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