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Genetics

Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules

(polysaccharide/nodulation/Calcofluor)

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ABSTRACT **By screening with the fluorescent stain Calcofluor, we have isolated 26 independent transposon Tn*5* insertion mutants of *Rhizobium meliloti* that are deficient in the production of a known extracellular polysaccharide (Exo$^-$). The mutants belonged to six distinct genetic groups based on the ability of their Exo$^-$ phenotype to be complemented by different recombinant plasmids from a *R. meliloti* clone bank. With few exceptions, all of the mutants formed ineffective (non-nitrogen-fixing) nodules on alfalfa. For all but one group, the complementing plasmids restored effective nodulation. These results establish a firm and extensive correlation between the ability of *Rhizobium* to produce a particular polysaccharide and symbiotic proficiency. The ineffective nodules appeared to contain no bacteroids and to form without shepherds' crooks or infection threads; this symbiotic phenotype matches that described for a set of independently isolated mutants that belong phenotypically and genetically to the group B exopolysaccharide mutants described previously [Finan *et al.* (1985) *Cell* 40, 869–877]. Apparently the exopolysaccharide, although not required for nodule formation, is involved in wild-type nodule invasion.**

The interactions between rhizobia and legumes that result in the development of nitrogen-fixing root nodules entail a complex series of events (1–3). Bacteria attach to a root hair, which curls to form a shepherd's crook. Bacterial penetration of the root hair occurs and a tubular infection thread forms, which carries the invading bacteria toward the base of the root hair. Cells deeper in the root cortex divide, and the bacteria are eventually released from the infection thread into the newly formed nodule cells. Nitrogen fixation occurs in the mature intracellular bacteria, called "bacteroids."

Recently it has become clear that the events of nodule formation (the development of root cortical tissue into a differentiated nodule structure) can be uncoupled from the events of nodule invasion (shepherd's crook formation, infection thread formation, and entry of bacteria into host cell cytoplasm). Strains of *Agrobacterium tumefaciens* and *Escherichia coli* containing *Rhizobium meliloti* nodulation genes (4–7) and a set of *R. meliloti* mutants at a locus that is also involved in extracellular polysaccharide synthesis (this paper; ref. 8) all form empty nodules (containing no intracellular bacteroids) without (or before) the development of shepherd's crooks or infection threads. It was recently reported that root cortical cell division in soybean can occur independent of infection thread formation, root hair curling, or even bacterial attachment (9, †).

A possible role for *Rhizobium* polysaccharides in the nodulation process has been a subject of great interest for a number of years (11, 12) but has not been demonstrated conclusively (13), although a single mutant that produced no water-soluble polysaccharide and formed ineffective (non-nitrogen-fixing) nodules was reported recently (14). In this paper we describe the isolation of an extensive set of mutants of *R. meliloti* that, though genetically diverse, all fail to produce a particular extracellular polysaccharide (exopolysaccharide, EPS). These mutants form ineffective nodules, thus establishing a firm correlation between the ability of *Rhizobium* to produce the polysaccharide and the ability to form a normal nitrogen-fixing nodule. The nodulation function associated with the polysaccharide appears to be in nodule invasion as opposed to nodule formation. A preliminary report has appeared (15).

MATERIALS AND METHODS

Genetic Manipulations. Bacterial strains and plasmids are listed in Table 1. LB and M9 media were as described (21), except that M9 medium contained 1 mmol of $MgSO_4$, 0.25 mmol of $CaCl_2$, and 1 mg of biotin per liter. Nitrogen-free M9 medium omitted the NH_4Cl. Liquid cultures of *E. coli* were routinely grown in LB medium and *R. meliloti* was grown in LB medium supplemented with $CaCl_2$ (2.5 mM) and $MgSO_4$ (2.5 mM). Transposon Tn*5* insertions in *R. meliloti* were made by mating *E. coli* strain 1830 or strain MM294A (pRK602) with *R. meliloti* strain Rm1021, RM5000, or Rm3357. Neomycin-resistant *R. meliloti* arose at a frequency of 10^{-5} with strain 1830 and 10^{-4} with strain MM294A (pRK602). Antibiotic levels used were streptomycin sulfate, 500 μg/ml, rifampin, 50 μg/ml, and neomycin sulfate, 200 μg/ml. Exopolysaccharide-deficient (Exo$^-$) mutants were obtained by screening for lack of fluorescence on LB agar containing 0.02% Calcofluor white M2R (Cellufluor, Polyscience, Warrington, PA), using a hand-held UV lamp (longwave). To test for cotransduction of Tn*5* with EPS deficiency, neomycin resistance was transduced (22) and colonies were screened for fluorescence on LB agar with Calcofluor. Recombinant plasmids that complemented the Exo$^-$ mutants were isolated from an *R. meliloti* clone bank on cosmid pLAFR1 obtained from Fred Ausubel. A triparental mating was performed [MM294A (pRK2013) × HB101 (pLAFR1) × *R. meliloti* Exo$^-$] and colonies were screened for fluorescence on LB or M9/glucose agar containing Calcofluor. Tetracycline HCl was used at a concentration of 10 μg/ml.

Detection, Isolation, and Analysis of EPS. EPS production in liquid culture was achieved by overnight incubation of the culture in nitrogen-free M9 medium. EPS was detected by adding Calcofluor to 0.02%, centrifuging on an Eppendorf centrifuge for 1 min, and viewing the supernatant and pellet under UV light. To detect hexadecyltrimethylammonium

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Abbreviation: EPS, exopolysaccharide.

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†Calvert, H. E., Malik, N., Mills, K. K., Pence, M. K. & Bauer, W. D. (1984) Second International Symposium on the Molecular Genetics of the Bacteria–Plant Interaction, June 4–8, Cornell Univ., Ithaca, NY, abstr. 90.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source or ref.
R. meliloti strain		
SU47	Wild type	16
Rm1021	Sm^r derivative of SU47	F. Ausubel
Rm5000	Rif^r derivative of SU47	T. Finan
Rm3357	SU47 *str-3 spc-1 rif-1 his-39 leu-53 nov-57*	17
E. coli strain		
1830	*pro met nal* (pJB4JI)	18
MM294A	*pro-82 thi-1 endA1 hsdR17 supE44*	G. Walker
HB101	*rpsL20* (Sm^r) *proA2 recA13 hsdS20* (rB$^-$, mB$^-$) *supE44*	G. Walker
Plasmid		
pJB4JI	pPH1::Mu::Tn*5*, contains Gm	18
pRK2013	Nm^r, ColE1 replicon containing transfer functions of RK2	19
pRK602	pRK2013 nm::Tn*9* containing Tn*5*	T. Finan
pLAFR1 clone bank	Tc^r, *R. meliloti* genes cloned onto cosmid vector pLAFR1	20
pD34	Exo$^-$ group A-complementing plasmid from clone bank	This work
pD2	Exo$^-$ group B-complementing plasmid from clone bank	This work
pD15	Exo$^-$ group C-complementing plasmid from clone bank	This work
pD5	Exo$^-$ group D-complementing plasmid from clone bank	This work
pD82	Exo$^-$ group B- and F-complementing plasmid from clone bank	This work

Sm^r, streptomycin resistant; Rif^r, rifampin resistant; Nm^r, neomycin resistant; Tc^r, tetracycline resistant; Gm^r, gentamycin resistant.

bromide (cetrimide)-precipitable material the culture was centrifuged and cetrimide (0.3 part of a 1% solution) was added slowly to the supernatant while stirring. A large-scale isolation of the polysaccharide was achieved by cetrimide precipitation (23) after a 2-day incubation of *R. meliloti* Rm1021 in nitrogen-free M9 medium. The yield was 0.25 mg/ml of culture supernatant.

For determination of monosaccharide composition, the EPS was hydrolyzed in 2 M HCl for 5 hr at 100°C. The hydrolysate was dried under a vacuum in the presence of NaOH pellets to remove HCl, redissolved in water, and dried again. Sample dissolved in water was applied to a cellulose thin-layer chromatography sheet, developed with 1-butanol/pyridine/water (6:4:3), and visualized with a spray of 1.23% *p*-anisidine/0.166% phthalic acid in ethanol.

Proton NMR spectroscopy was done on a Bruker WM250 Fourier-transform instrument at 80°C. Sample (40 mg of dry weight) dissolved in water was sonicated to aid in dissolution and decrease viscosity. The solvent was ^2H_2O, and the chemical-shift standard was sodium 3-(trimethylsilyl)-1-propanesulfonate (trimethylsilyl protons = 0 ppm). Fluorescence microscopy was done with an Olympus model BH fluorescence microscope, using exciter filter UG1 and barrier filter L420.

Phage Sensitivity Tests. Phage resistance patterns were determined in spot tests (24). The absence of a spot or the presence of a turbid spot indicated resistance. Calibration of the spot tests with a representative of each mutant group showed that resistance corresponded to plaquing efficiencies (titering method of ref. 22) of $<10^{-6}$, except that ϕM5 formed plaques on the groups B and E mutants with efficiencies of 10^{-3} to 10^{-4}.

Plant Nodulation Tests. Plants were grown on Petri dishes of Jensen's agar (25). A notch was melted into the edge of the Petri dish through which the plant emerged. Seeds of *Medicago sativa* cv. Iroquois were surface sterilized in a 1:1 mixture of water/5.25% sodium hypochlorite (Clorox) for 20 min. Seeds of *Melilotus alba* (a gift from Fred Ausubel) were surface sterilized in concentrated H_2SO_4 for 10 min. Nitrogen fixation was determined by plant growth and acetylene reduction by excised nodules. The bacterial content of nodules was determined after surface sterilization in 20% (vol/vol) Clorox for 2 min, followed by rinsing in water and LB medium and crushing in LB medium containing 0.3 M glucose.

RESULTS

Isolation and Genetic Characterization of Mutants. Colonies of *R. meliloti* strains Rm1021 and Rm5000 grown on LB agar containing Calcofluor exhibited a blue-green fluorescence when irradiated with long-wave UV light (8), as reported for the related bacterium *A. tumefaciens* (26). The fluorescence was also apparent on M9/glucose, M9/galactose, and M9/succinate agar containing Calcofluor. By screening random Tn*5* insertion mutants on LB/Calcofluor agar, we isolated 26 mutants that produced nonfluorescent colonies ("Calcofluor-dark", ref. 8); these were found at a frequency of ≈1 for every 3000 neomycin-resistant colonies. In the majority of cases, the Calcofluor-dark phenotype was cotransducible with neomycin resistance of Tn*5* (Table 2). As discussed below, these mutants appear to be defective in the production of the major acidic EPS of *R. meliloti*.

A *R. meliloti* cosmid clone bank was conjugally transferred into the mutants Rm7061, Rm7034, Rm7002, Rm7082, Rm7015, and Rm7005, and strains containing a complementing recombinant plasmid were detected by their fluorescence on LB or M9/glucose agar containing Calcofluor. We were able to classify the mutants into six distinct genetic groups on the basis of their ability to be complemented by five different recombinant plasmids (Table 2). Plasmids pD34, pD2, pD15, and pD5 complemented the mutants in groups A, B, C, and D, respectively. Plasmid pD82 complemented the mutants in

Table 2. Exo$^-$ mutants of *R. meliloti*: Complementation by recombinant plasmids from a *R. meliloti* cosmid clone bank

Group A	Group B	Group C	Group D	Group E*	Group F
		Rm1021 background			
Rm7061	Rm7094	Rm7020	Rm7017	Rm7022	Rm7055
Rm7034	Rm7082	Rm7027	Rm7043	Rm7029	Rm7056
Rm7023			Rm7053		
Rm7031			Rm7054		
Rm7032					
		Rm5000 background			
Rm7011	Rm7002	Rm7015	Rm7005†	Rm7006†	
Rm7009	Rm7013				
Rm7016	Rm7014				

Tn*5* cotransduces with EPS deficiency except where noted. Strains Rm7061, Rm7034, Rm7023, Rm7094, Rm7082, Rm7020, Rm7027, Rm7017, Rm7043, Rm7022, and Rm7029 were isolated by crossing *E. coli* strain 1830 with *R. meliloti* strain Rm3357 and subsequently transducing neomycin resistance into strain Rm1021. Strains Rm7002, Rm7005, and Rm7006 were obtained by crossing *E. coli* strain 1830 with *R. meliloti* strain Rm5000. Strains Rm7031, Rm7032, Rm7053, Rm7054, Rm7055, and Rm7056 were obtained by crossing *E. coli* strain MM294A (pRK602) with strain Rm1021. Strains Rm7011, Rm7009, Rm7016, Rm7013, Rm7014, and Rm7015 were obtained by crossing *E. coli* strain MM294A (pRK602) with strain Rm5000.

*Not complemented.

†Tn*5* does not cotransduce with EPS deficiency.

both groups B and F, an observation suggesting that the two groups are genetically linked. The plasmids appeared by restriction endonuclease analysis to carry distinct segments of *R. meliloti* DNA, except that pD2 (group B) and pD82 (groups B and F) had *Eco*RI and *Bam*HI fragments of the same size. Group E was defined as the set of mutants that could not be complemented by these five plasmids or by any other cosmid we could isolate from the clone bank.

With the exception of the group D mutants, the Calcofluor-dark mutants seemed to be completely defective in the production of the Calcofluor-binding material and did not fluoresce at all either on LB/Calcofluor agar or on M9/glucose/Calcofluor agar. In contrast, the group D mutants appeared to have a less extreme defect since they exhibited a slight fluorescence on LB/Calcofluor agar and a fluorescence similar to wild-type Rm1021 on M9/glucose/Calcofluor agar. In addition, the group D-complementing plasmid enhanced the fluorescence of the wild-type strain Rm1021 as well as of the group D mutants; therefore, the results obtained to date with this particular plasmid do not distinguish between true complementation and compensation by overproduction.

The classification of the Calcofluor-dark mutants into the above six groups was originally based on the ability of particular recombinant plasmids to complement their defects in fluorescence on Calcofluor-containing medium. However, we also found that the patterns of resistance of these mutants to nine *R. meliloti* bacteriophage (ϕM1, -5, -6, -7, -9, -10, -11, -12, and -14) were entirely consistent with this grouping. Thus, the mutants belonging to groups A, D, and F were resistant to none of the phages, the groups B and E mutants were resistant to four (ϕM5, -9, -10, and -14), and the group C mutants were resistant to all nine. These intergroup differences in phage sensitivities indicate that, despite similar phenotypes with respect to EPS production and symbiosis, the groups differ with respect to surface properties. The phage resistance results were also of interest since we had classified mutants as belonging to group E solely on the basis of their inability to be complemented by any cosmid present in our bank. However, the fact that all of the mutants in this group have the same pattern of phage resistance suggests that they may indeed be related.

Interestingly, the resistance pattern of the group B mutants matched the previously established pattern of a set of Fix$^-$ mutants isolated independently as spontaneous mutants resistant to particular antibodies or phage (24). These were mapped to a single locus and were later found to possess the Calcofluor-dark phenotype and to belong to group B (8).

Calcofluor-Dark Mutants Are Defective in EPS Production. We have obtained evidence suggesting that the Calcofluor-dark mutants are defective in the synthesis of the major acidic EPS of *R. meliloti*. This acidic EPS (Fig. 1) (23, 27) has been isolated previously from the culture supernatant of Rm1021,

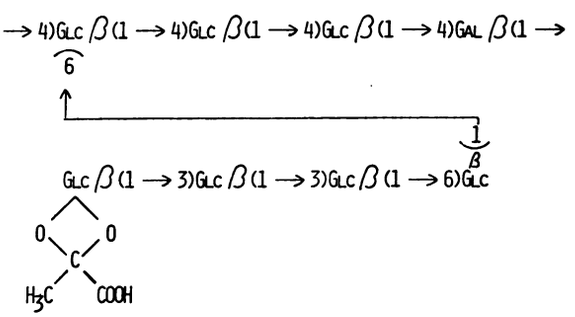

FIG. 1. Reported structure of the *R. meliloti* acidic EPS (23, 27). *O*-succinyl and *O*-acetyl groups are also present at undetermined sites (28, 29).

the strain used in this study, by precipitation with the cationic detergent cetrimide (23). Similarly, we were able to use cetrimide to precipitate a large amount of material from the culture supernatant of the wild-type strain Rm1021 incubated in nitrogen-free M9/glucose medium; this material fluoresced under long-wavelength UV in the presence of Calcofluor and contained an abundance of fibrillar material, as observed by fluorescence microscopy (data not shown). ^{1}H NMR spectroscopy (Fig. 2) showed that a substance very similar to the previously described EPS was at least a major component of our cetrimide-precipitated material. Furthermore, acid hydrolysis of this material followed by thin-layer chromatography showed only an intense spot that comigrated with glucose and a less intense spot that comigrated with galactose, as would have been expected from the published structure of the EPS. Under identical conditions to those used to obtain the cetrimide precipitate from the wild-type strain, we obtained no precipitate from representative mutants of the Calcofluor-dark groups A, B, C, E, and F (Rm7061, Rm7094, Rm7015, Rm7006, and Rm7055, respectively). A small amount of precipitate was obtained from the incompletely dark group D mutant Rm7017. These observations suggest that all of the mutants that we have identified on the basis of their Calcofluor-dark phenotype have defects in the production of the major *R. meliloti* acidic EPS. We therefore use the phenotypic designation Exo$^-$ to refer to these mutants.

In the course of these studies, we also observed that the production of the *R. meliloti* acidic EPS is apparently regulated in response to physiological conditions. Although the wild-type strain Rm1021 produced Calcofluor-staining material on M9/glucose agar, in liquid M9 medium it only produced Calcofluor-staining material after overnight incubation in the absence of nitrogen, sulfur, or phosphorus. The same conditions were required for the production of cetrimide-precipitable material.

Formation of Ineffective Nodules by the Exo$^-$ Mutants. The wild-type strains Rm1021 and Rm5000 formed effective (nitrogen-fixing) nodules on *M. sativa* and *M. alba* within a

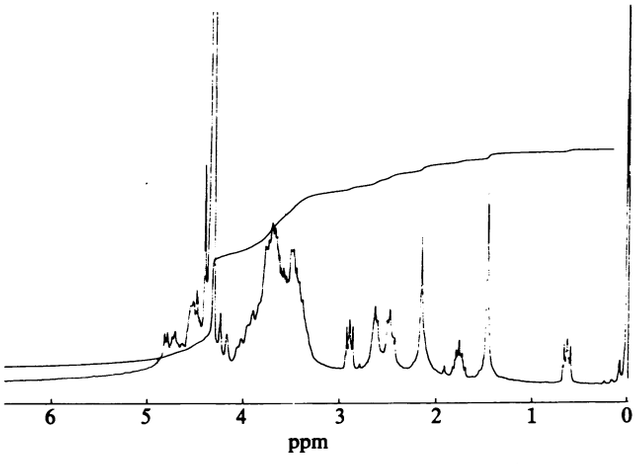

FIG. 2. Proton NMR spectrum of cetrimide-precipitated EPS from *R. meliloti* strain Rm1021, showing signals corresponding to those reported (23). The singlets at 1.46 and 2.15 ppm represented the methyl protons of the 1-carboxyethylidine and acetoxyl groups, respectively. The multiplets at 2.48 and 2.63 ppm represented the methylene protons of the succinyl group. The complex regions from 3.3 to 4.0 and 4.5 to 4.9 ppm represented ring protons and β-anomeric protons, respectively, of the sugar constituents. The remaining signals were the solvent peak (^{1}H^{2}HO, 4.31 ppm) and its spinning side bands (4.17, 4.23, 4.40, and 4.48 ppm) and the chemical-shift standard sodium 3-(trimethylsilyl)-1-propanesulfonate (0.00, 0.63, 1.76, and 2.89 ppm).

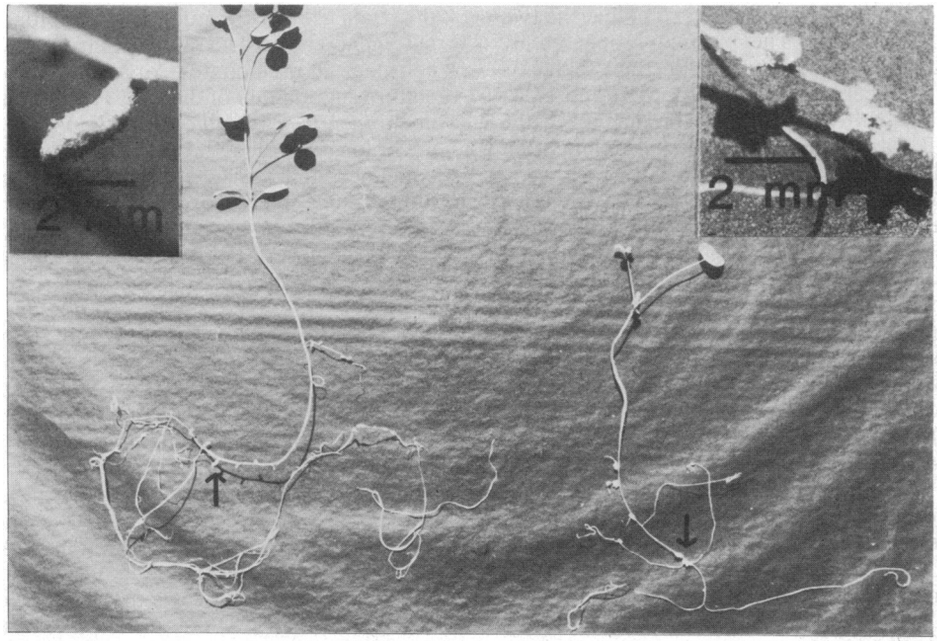

FIG. 3. Nodules formed by wild-type strain Rm1021 (*Left Inset*) and a representative EPS-deficient mutant (Rm7017, *Right Inset*) shown 30 days after inoculation. Nodules indicated by arrows are shown in *Insets*.

week after inoculation. Approximately 10 nodules of the typical pink, cylindrical appearance were present per plant after 3 weeks. In contrast, all of the Calcofluor-dark Exo^- mutants described above formed round, white, ineffective nodules (blanched, stunted plants and no detectable acetylene reduction) on *M. sativa* (Fig. 3), with the exceptions of two of the incompletely dark group D strains (Rm7053 and Rm7054), which formed nodules that were indistinguishable from those formed by wild-type Rm1021. Similarly, representatives of each mutant group formed round, white, ineffective nodules on *M. alba*. The nodules induced by the mutants on *M. sativa* remained white and ineffective for at least 6 weeks (until the agar growth medium dried up), except that the nodules formed by the group F strains Rm7055 and Rm7056 sometimes turned pink and began to acquire a cylindrical shape after 3–6 weeks, at which time they were effective as determined by acetylene reduction and renewed growth of the plant. Nodules induced by mutants in groups A, B, D, and F first appeared at about the same time as nodules induced by wild-type Rm1021 or Rm5000 but tended to exceed the wild-type nodules in number after 3 weeks. Nodules induced by mutants in groups C and E appeared several days later and were fewer in number. Members of groups A–C and F, containing a complementing plasmid, formed normal, effective nodules on *M. sativa*. The group D mutants Rm7017 and Rm7043 formed only white, ineffective nodules even when containing the recombinant plasmid that suppressed the defect in fluorescence in Calcofluor-containing medium.

The ineffective nodules induced by our Calcofluor-dark Exo^- mutants were similar to those induced by the set of independently isolated spontaneous mutants mentioned above, which were found to be Exo^- (Calcofluor-dark) and to belong to group B. Thus, in contrast to effective nodules, crushing of the nodules formed by our mutants on *M. sativa* released few, if any, bacteria as judged by light microscopy and plating for colonies. Those few bacteria that were observed presumably existed superficially and intercellularly as reported (8). More detailed observations by Ann Hirsch (personal communication) confirmed that the nodules contained no intracellular bacteroids and showed that, although the bacteria appeared to attach to the root hairs, the formation of shepherds' crooks and infection threads was absent or delayed. Nodules that were quite old sometimes developed infection threads secondarily. The nodules were organized differentiated structures with a distal meristem, a distinct endodermis that separated the nodule cortex from the main body of the nodule, and peripheral vascular bundles; they were not the tumor-like structures that have been reported (30, 31).

DISCUSSION

Our finding that a genetically diverse but phenotypically homogeneous set of Exo^- mutants is almost uniformly Fix^- (forming non-nitrogen-fixing nodules) suggests that the *R. meliloti* acidic EPS is intimately involved in the formation of an effective nodule. Among our completely Calcofluor-dark mutants (groups A–C, E, and F), the only deviations from the strictly Fix^- phenotype occur with the two group F strains Rm7055 and Rm7056, which form ineffective nodules that sometimes become effective only after a long delay. Furthermore, recombinant plasmids that complement the defects of groups A–C and F mutants in Calcofluor fluorescence also restore their Fix^+ phenotype. The simplest interpretation of this set of observations is that the symbiotic deficiency of the Exo^- mutants described in this paper is a consequence of their deficiencies in polysaccharide production.

Since the ineffective nodules formed by the Exo^- mutants appear similar in their absence of shepherds' crooks, infection threads, and bacteroids to those formed by a set of independently isolated group B mutants (8), the defect associated with the polysaccharide deficiency seems to be in nodule invasion as opposed to nodule formation. There are several possible roles of the EPS in the invasion process. The polysaccharide might serve as a signal for the induction of a step in the invasion process, such as shepherd's crook or infection thread formation. Several reports have presented evidence that rhizobial extracellular factors promote root hair deformation or infection thread formation (32–35). Alternatively, the polysaccharide might be a structural component of the nodule, possibly the infection thread matrix, which has been suggested to be of rhizobial origin on the basis of its staining properties (see ref. 2). It will be interesting to see if

the infection threads that sometimes form secondarily contain a normal thread matrix.

The precise nature of the molecular defects of the Exo$^-$ mutants described in this paper remains to be established. As we have discussed, a distinguishing phenotype of these mutants is that they are defective in the production of the major acidic EPS of *R. meliloti*. Since the Exo$^-$ mutants were all identified because of a deficiency in the production of Calcofluor-staining material it seems likely that at least some of the Exo$^-$ mutations are in genes coding for proteins involved in the biosynthesis of the acidic polysaccharide. Some other mutations may cause more general alterations in the cell membrane or cell surface that interfere indirectly with EPS production; the group C mutants, which are resistant to the nine *R. meliloti* phage tested, may possibly have this type of defect. The nature of the defects in the group D mutants is unclear. These mutants exhibit a slight Calcofluor fluorescence and produce a small amount of cetrimide-precipitable material. However, two of the group D mutants give rise to effective nodules, whereas two others form ineffective nodules even when a recombinant plasmid that enhances EPS production is present. The symbiotic deficiency of these latter two mutants could arise from an effect that is distinct from their partial EPS deficiency, such as a membrane defect.

A great variety of polysaccharides is produced by various microbes, many of which are of known importance in agriculture, medicine, and industry (36–39). Nevertheless, the biosynthesis of many of these polysaccharides has not been characterized. A preliminary biochemical study of the synthesis of the *R. meliloti* acidic EPS has appeared (10), but, to our knowledge, the genetic characterization of a known rhizobial polysaccharide has not been reported previously.

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