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NOTE

Exopolysaccharide-Deficient Mutants of *Rhizobium fredii* HH303 Which Are Symbiotically Effective

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Nineteen Tn*5*-induced mutants of *Rhizobium fredii* HH303 defective in acidic exopolysaccharide synthesis were isolated by screening for lack of Calcofluor fluorescence. They were grouped by complementation analysis by using *Rhizobium meliloti* cosmids carrying *exo* genes. All of the 19 mutants were symbiotically effective or partially effective, indicating that the major bacterial acidic exopolysaccharide of this strain of *R. fredii* may not be required for symbiotic development in the soybean.

Extracellular polysaccharides (EPS) are generally considered to play an important role in the development of nitrogen-fixing nodules on legumes. Some *Rhizobium* and *Bradyrhizobium* mutants deficient in extracellular polysaccharide production are unable to nodulate (7), some are unable to fix nitrogen (4, 5, 13), and some are unaffected in symbiotic nitrogen fixation (2, 18). The acidic EPS of *Rhizobium meliloti* and *Rhizobium phaseoli* reacts with Calcofluor (3, 13, 19), a fluorescent stain for β-linked polysaccharides (14), and lack of fluorescence (Calcofluor dark) has been used to identify EPS$^-$ mutants in *R. meliloti* and an *Agrobacterium* sp. (3, 13).

R. fredii is a fast-growing soybean microsymbiont (8, 10). The species is physiologically more similar to other fast-growing *Rhizobium* species than to *Bradyrhizobium japonicum*. We found that among 14 wild-type *R. fredii* strains tested, two (HH103 and HH303) were Calcofluor positive. Strain HH303 was selected for mutagenesis, and Calcofluor-dark mutants were found to be defective in acidic EPS production; however, unlike the mutants of *R. meliloti*, they were symbiotically effective with soybean plants.

Bacterial strains and plasmids. *R. fredii* strains shown in Table 1 were obtained from H. H. Keyser of this laboratory. *R. fredii* strains were routinely cultivated in YEM or M9 medium (15). Five *R. meliloti* Exo plasmids, pD2, pD5, pD15, pD34, and pD56 (pD82), complementing the *R. meliloti* Exo$^-$ mutants in groups B, D, C, A, and B and F, respectively, were obtained from G. C. Walker of the Massachusetts Institute of Technology, Cambridge, Mass. (13). The plasmids (Tc^r) were maintained in *E. coli* HB101 (*pro leu thi lacY str endI recA hsdR hsdM*) (15). *E. coli* was grown in LB medium (15).

To screen for Calcofluor-positive strains, the 14 *R. fredii* strains were streaked on YEM agar plates supplemented with 0.02% Calcofluor white M2R (Sigma Chemical Co., St. Louis, Mo.), pH 7.2, and incubated for 3 days at 28°C. The growing colonies were examined for fluorescence under short UV light. Two strains, HH103 and HH303, induced yellow-green fluorescence, whereas the other 12 strains were nonfluorescent (Table 1). These two strains induced effective nodules on *Glycine max* cv. Williams, whereas the strains ineffective on Williams were Calcofluor dark. This suggests that the structure or composition of EPS of these two strains may be different and may be involved as a host specificity determinant, even though two other effective strains, USDA 191 and HH003, appeared Calcofluor dark. Strain HH303 induced fluorescence on YEM, TY (1), M9 with glucose, M9 with mannitol, M9 with fructose, and M9 with succinate agar plates containing Calcofluor.

Isolation and characterization of Exo$^-$ mutants of strain HH303. Transposon Tn*5* mutagenesis of *R. fredii* HH303 was performed as described previously (11). Nineteen Exo$^-$ mutants were isolated as Calcofluor-dark colonies on YEM agar plates supplemented with Calcofluor as described by Leigh et al. (13). Calcofluor-dark colonies were detected at a frequency of about 10^{-3} among the kanamycin-resistant colonies.

The mutants were classified by complementation analysis for Calcofluor fluorescence with *R. meliloti* Exo plasmids. Each plasmid in *E. coli* HB101 was transferred to the Exo$^-$ mutants of *R. fredii* HH303 by triparental mating by using pRK2073 as a helper plasmid (6). The mutants which became fluorescent by introduction of the same Exo plasmid were grouped together (Table 2). Four mutants were complemented by pD34 (group A), three were complemented by both pD2 and pD56 (group B), and seven were complemented by pD56 (group F). Five mutants were not complemented by any Exo plasmids. None of the mutants was complemented by plasmids carrying *exoC* or *exoD* genes which are located on the *R. meliloti* chromosome.

Isolation and analysis of EPS. EPS produced by the wild-type strain and Exo$^-$ mutants was isolated as described previously (12). Cells grown in M9 medium with glucose were harvested during the logarithmic phase of growth, suspended in 100 ml of nitrogen-free M9 medium with glucose to an optical density of 0.5 at 650 nm, and incubated at 28°C for 2 days. Cell number was determined spectrophotometrically with a conversion factor of optical density 1 equals 7.6×10^8 cells/ml. The supernatant was collected after centrifugation of the cell culture and freeze-dried. The

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TABLE 1. *R. fredii* strains tested for fluorescence with Calcofluor[a]

Strain	Effective symbiosis with soybean cv.[b]:		Calcofluor fluorescence
	Peking	Williams	
USDA 191	+	+	−
USDA 192	+	−	−
USDA 193	+	−	−
USDA 194	+	−	−
USDA 201	+	−	−
USDA 205	+	−	−
USDA 206	+	−	−
USDA 208	+	−	−
USDA 214	+	−	−
USDA 217	+	−	−
USDA 257	+	−	−
HH003	+	+	−
HH103	+	+	+
HH303	+	+	+

[a] Tested on YEM agar plates containing 0.02% Calcofluor.
[b] See references 8 and 10.

dried pellet was dissolved in 3.5 ml of water and centrifuged to remove undissolving material. Ethanol was added to 90%, and the precipitated EPS was recovered by centrifugation. The EPS pellet was air-dried overnight and dissolved in 1 mM KCl for analysis by liquid chromatography.

The EPS sample (1 ml) was loaded onto a DEAE-cellulose column (1.5 cm × 6.5 cm) equilibrated with 1 mM KCl and then washed with 100 ml of 1 mM KCl to elute neutral EPS, which was less than 10% of the total EPS. The acidic EPS which bound to the column was eluted with 80 ml of a linear gradient, 1 mM to 1 M KCl. Fractions (3.0 ml) were tested for glucose equivalents by the anthrone assay. The acidic EPS from the wild-type strain or mutants carrying the complementing Exo plasmids of *R. meliloti* eluted at about 0.7 M KCl, whereas no acidic EPS was detected from the samples of Exo$^-$ mutants (Fig. 1A and C).

Plant assay of Exo$^-$ mutants. Plant tests were done with soybeans (*Glycine max* cv. Williams and cv. Peking) in Leonard jars filled with vermiculite (11). Symbiotic phenotypes were determined by nodule color and morphology, plant shoot color and weight, and by assaying for acetylene reduction.

The symbiotic phenotypes of the 19 Exo$^-$ mutants with *G. max* cv. Williams is illustrated in Table 3. With few exceptions, the mutants induced normal nodules with the characteristic pink color of the wild-type strain and were symbiotically effective. RfK1010 and RfK1033 induced small pink nodules, in addition to normal nodules, and were partially

TABLE 2. Complementation group of Tn5-induced Exo mutants of *R. fredii* HH303

Exo group[a]	Mutants
A	RfK1004, RfK1009, RfK1021, RfK1023
B	RfK1001, RfK1017, RfK1022
F	RfK1003, RfK1012, RfK1013, RfK1018, RfK1026, RfK1027, RfK1029
Unknown[b]	RfK1002, RfK1010, RfK1011, RfK1028, RfK1033

[a] Determined by complementation analysis with five *R. meliloti* Exo plasmids, pD34 (group A), pD2 (group B), pD15 (group C), pD5 (group D), and pD56 (group B and F) (13).
[b] Not complemented by any *R. meliloti* Exo plasmid.

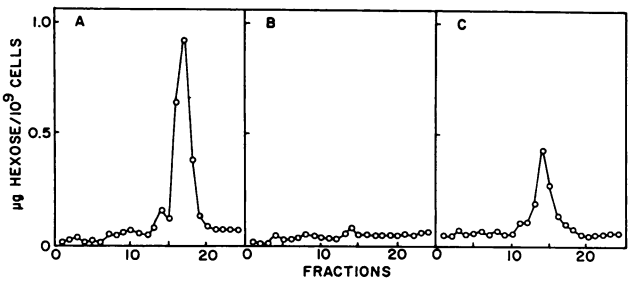

FIG. 1. Elution profile of acidic EPS from a DEAE-cellulose column. Amount of hexose in each fraction was determined by the anthrone assay (9). (A) Wild-type strain; (B) RfK1017; (C) RfK1017 complemented with pD2 (*exoB*).

effective. Symbiotic development may be delayed or partially inhibited in these mutants. Bacteroids were recovered from the nodules as described previously (11) and were tested to ensure that they retained the kanamycin resistance conferred by Tn*5* and that they were Calcofluor dark. Similar results were observed in plant tests with *G. max* cv. Peking (data not shown).

Two very recent reports indicating that *R. meliloti* may synthesize two different acidic extracellular polysaccharides has complicated the interpretation of the role of the microbial EPS in nodule development. Kondorosi and co-workers for years have been using *R. meliloti* strain AK631, which is a small colony derivative of strain 41. There are no recognized differences in symbiotic effectiveness between the two strains. Recently, Putnoky et al. (16) recognized that strain 41 is Calcofluor bright, while AK631 is Calcofluor dark. They suggested that AK631 did not produce acidic EPS, but no data has been published. Another observation that influences the interpretation of results with EPS-deficient mutants is that of Reed et al. (17). They recently reported that

TABLE 3. Symbiotic phenotype of Exo$^-$ mutants[a]

Strain (RfK)	Nodules[b]		Shoot[c]		ARA[d]	Effective symbiosis
	No.	Color	Dry wt	Leaf color		
HH303	10	P	1.8	G	8.1	+
None	0		1.3	Y	0.6	−
1001	16	P	2.0	G	6.0	+
1002	15	P	1.6	G	5.4	+
1003	13	P	2.1	G	3.7	+
1004	10	P	2.4	G	4.3	+
1009	17	P	1.8	G	12.3	+
1010	12	P	1.4	G/Y	3.2	+/−
1011	10	P	2.4	G	6.0	+
1012	19	P	1.5	G	10.5	+
1013	9	P	1.5	G	4.3	+
1017	8	P	2.1	G	4.2	+
1018	12	P	1.7	G	8.6	+
1021	14	P	1.9	G	5.0	+
1022	17	P	2.5	G	4.1	+
1023	15	P	1.6	G	6.0	+
1026	10	P	2.2	G	4.8	+
1027	17	P	1.5	G	9.5	+
1028	10	P	1.5	G	6.5	+
1029	9	P	1.4	G	5.5	+
1033	12	P	1.3	G/Y	3.3	+/−

[a] Results are averages of data from two plant tests with *Glycine max* cv. Williams.
[b] Nodule number per plant. Color: P, pink; W, white.
[c] Dry weight in grams per plant. G, Green; Y, yellow.
[d] Acetylene reduction activity, micromoles of C_2H_4 per h per plant.

R. meliloti 1021 has the latent capacity to synthesize a second EPS (Calcofluor dark) which is different structurally from the EPS produced by the wild-type strain. This EPS could substitute for the wild-type Calcofluor-bright EPS in conferring the Fix^+ phenotype in nodulation. Whether small amounts of such an EPS is present in *R. meliloti* AK631 and in our mutants is a question which needs to be investigated.

It is interesting that the *exo* genes of *R. meliloti* which complement *R. fredii* Exo^- mutants are located in the megaplasmid, whereas *exo* genes which originated from the chromosome of *R. meliloti* (*exoC* and *exoD*) did not complement any Exo^- mutants of strain HH303.

Since some of the mutations in acidic EPS synthesis of *R. fredii* were complemented by *R. meliloti exo* genes, some of the steps in acidic EPS synthesis should be common between the two species. However, five mutants of strain HH303 were not complemented by any of *R. meliloti* Exo plasmids, and none of the mutants was complemented by ExoC or ExoD plasmids. Therefore, some genes involved in the acidic EPS synthesis must be unique in each species, and the structure of EPS from HH303 should be different from that of *R. meliloti*.

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