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Strains of *Rhizobium fredii* Effectively Nodulate and Efficiently Fix Nitrogen with *Medicago sativa* and *Glycine max*

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Abstract

Effective nodulation of and efficient symbiotic nitrogen fixation with alfalfa by strains of *Rhizobium fredii*, the fast-growing soybean microsymbiont, is herein reported. This discovery has broad implications for host specificity research and strain improvement. Effective nodulation of Egyptian alfalfa cultivar 'Giza 4' by *R. fredii* type strain USDA 205 was observed and deemed anomalous yet intriguing since nodulation of alfalfa by *R. fredii* had not been previously reported. To investigate this anomaly, twenty-two strains of *R. fredii*, including the eleven strains first isolated fifteen years ago from east central provinces of China, and three strains of *R. meliloti* were evaluated for symbiotic capabilities with 'ARC' alfalfa, a standard improved cultivar of *Medicago sativa*. Efficient nitrogen-fixing symbioses were formed by *R. fredii* USDA strains 201, 208, 209 and 214 with this cultivar and four other USDA strains of *R. fredii*, including the type strain USDA 205, formed inefficient nodules. The former strains produced high nodule numbers and high plant dry weights under conditions of nil combined nitrogen, and strains 201, 208 and 214 exhibited symbiotic nitrogen fixation activities comparable to

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those of strains of *R. meliloti*, the long-recognized nitrogen-fixing microsymbiont of alfalfa. *R. fredii* strains efficiently nodulating both soybean and alfalfa were confirmed by megaplasmid DNA content, by reinfection of both hosts, and by DNA RFLP.

Keywords: host specificity, Nod factors, *Sinorhizobium*, symbiosis, strain improvement

1. Introduction

Host specificity of legume microsymbionts is a subject of wide interest in the scientific community. *Rhizobium* sp. strain NGR234 has a broad host range for nodulation, but not for efficient nitrogen fixation, with more than 60 different legume genera and the non-legume *Parasponia* (Dénarié, 1993). *Rhizobium fredii* strain USDA 257 also has a broad host range since it nodulates a majority of these same genera (Krishnan and Pueppke, 1994). *Rhizobium* sp. (*Leucaena*) strain DS 65 was recently observed to nodulate several small- and large-seeded legume plant species (Swelim et al., 1996). Conversely *Rhizobium meliloti* strains have a narrow, restricted host range with species of *Medicago*, *Melilotus* and *Trigonella* (Jordan, 1984), but Gao and Yang (1995) recently reported a field isolate of *R. meliloti* that effectively nodulated soybean in China.

Rhizobium meliloti, which is now also known as *"Sinorhizobium" meliloti*, has a close phylogenetic relationship with *R. fredii*, or *S. fredii* (De Lajudie et al., 1994; Martinez-Romero and Caballero-Mellado, 1996). The nucleotide sequences of a particular 260-bp segment of their respective 16S rRNA genes were identical (Jarvis et al., 1992). A close phylogenetic relationship is also clearly indicated by serology (Sadowsky et al., 1987), by the fact that both bacterial species carry huge megaplasmids as large or larger than 1000 MDa (Hashem and Kuykendall, 1994; Hashem et al., 1996), and by the fact that some recently studied *R. fredii* phages infected some strains of *R. meliloti* (Hashem et al., 1996). We observed effective nodulation of Egyptian alfalfa cultivar 'Giza 4' by *R. fredii* type strain USDA 205 when evaluating putative exconjugants and parental strain controls in examining interspecific transfer of host specificity genes. Therefore, the main objective of this study was to examine a number of *R. fredii* strains for their ability to nodulate and fix nitrogen with a standard cultivar of *Medicago sativa*.

2. Materials and Methods

Rhizobium strains and host cultivars

All *Rhizobium* strains, except strain ARC 1, were from the USDA, ARS

Rhizobium Germplasm Resource Center, Beltsville, MD, USA. *R. fredii* USDA strains 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 214, 217, 257 and *R. meliloti* strains USDA 1936, 1954, and 1969 were used. *R. meliloti* strain ARC 1, from the *Rhizobium* Culture Collection of the Agricultural Research Center of Egypt, was previously characterized for plasmid content (Hashem and Kuykendall, 1994) and for symbiotic competence with alfalfa (Kuykendall et al., 1994). *Bradyrhizobium japonicum* strain I-110 ARS (Kuykendall and Weber, 1978) was used as a negative control. *Rhizobium* strains were grown in yeast extract mannitol broth (Vincent, 1970) on a rotary shaker at 30°C to early log phase for individual use as seed inoculants. Strains were maintained by storage in 50% sterile glycerol at –70°C.

Medicago sativa L. cv. 'ARC' is a USDA-bred cultivar with multiple pest resistance (Devine et al., 1975). Seeds of *M. sativa* cv. 'Giza 4' were from the Field Crop Research Institute of the Agricultural Research Center of Egypt. *Glycine max* L. (Merr.) cv. 'Peking' seeds were from P. B. Cregan, USDA, ARS, Beltsville, MD, USA.

First effectiveness test

At Tanta University in Egypt, seeds of *Medicago sativa* cv. 'Giza 4' were surface-sterilized (Vincent, 1970), planted in 50 × 5-cm glass tubes containing nitrogen-free soft agar medium (Abdel-Gaffar and Jensen, 1966) and inoculated with *R. meliloti* strain ARC 1 or *R. fredii* strain USDA 205. Tubes containing uninoculated seeds served as controls. Growth tubes were arranged randomly with four replications of each treatment. At six weeks after planting, plants were removed from the tubes, the tops separated from the roots, and the nodules counted. Plant tops were dried at 70°C, weighed and ground in a Wiley mill to < 0.1 mm particle size. Plant nitrogen content was determined by semi-micro Kjeldahl analysis (Bradstreet, 1965).

Second effectiveness test in the greenhouse

At the Beltsville Research Center in the U.S., the ability of 22 *R. fredii* strains to nodulate and fix nitrogen with *M. sativa* cv. 'ARC' was examined in the greenhouse during January and February, 1996. Surface-sterilized seeds of *M. sativa* were planted in autoclaved Leonard jars (Leonard, 1943) containing vermiculite and N-free plant nutrient solution (Norris, 1964). The seedlings were thinned to ten per jar and each jar received three milliliters of an early log phase culture of each strain. There were four replicate jars of each treatment. Four jars were left uninoculated as controls. Jars were arranged in the greenhouse in a randomized complete design. After eight weeks, plants

were harvested, roots were separated from the tops, nodule number was determined, and nitrogenase activities were measured (Sloger, 1969). Plant tops were subjected to drying and analysis as described above. Strains which formed nodules on alfalfa plants were recovered as bacteriologically pure clones by surface-sterilization of the nodules with 3% H_2O_2 for 1 h, followed by three rinses in sterile distilled water. The nodules were aseptically crushed and some of the contents streaked on yeast extract mannitol agar medium (Vincent, 1970) containing 25 μg ml^{-1} Congo red as a differentiating agent.

Third effectiveness test

The third experiment was conducted to examine the ability of nodule reisolates to nodulate and fix nitrogen with both soybean and alfalfa. Reisolates obtained from root nodules of plants that had been inoculated with strains USDA 1936, USDA 201, USDA 208, or USDA 214 were individually used to inoculate 'ARC' alfalfa and soybean cv. 'Peking' in Leonard jars in a complete randomized block design consisting of two plant species, alfalfa and soybean, the three *R. fredii* strains previously determined as effective on alfalfa, one *R. meliloti* strain and uninoculated control. There were five replicates of each treatment. The tops of the jars were filled with vermiculite and the bottoms were filled with N-free plant nutrient solution (Norris, 1964). Jars were sterilized by autoclaving for two hours. Soybean and alfalfa seeds were surface-sterilized (Vincent, 1970) and planted, sufficient to establish four and ten plants, respectively, per Leonard jar. Each jar, except the uninoculated controls, received 3 ml of log-phase *Rhizobium* culture as an inoculum. Jars were placed in a growth chamber where the temperature was set at 25°C. Light and dark periods were 16 and 8 h, respectively. Plants were harvested four weeks after planting. Nodule number, top dry weight and nitrogenase activities were determined as described in the preceding sections.

Plasmid profile analysis

Healthy nodules were collected from the roots of alfalfa plants that had been inoculated with *R. meliloti* strain USDA 1936 and *R. fredii* USDA strains 205, 201, or 208. Nodules were surface-sterilized and streaked onto differentiation medium as described above. Plates were incubated at 30°C for five days. Nodule reisolates were examined for their plasmid DNA content in direct comparison with the respective original strain that had been used as inoculum according to our previously described method (Hashem and Kuykendall, 1994).

RFLP analysis

RFLP analysis of total DNA was conducted to verify the identity of nodule isolates in comparison with the original strains. Probe DNAs were isolated and purified by the method of Itoh et al. (1984), except that the *E. coli* cultures were grown in 2 × LB medium (Sambrook et al., 1989). Total DNAs from *Rhizobium* strains were isolated from 50-ml cultures grown to stationary phase in A1E medium (Kuykendall, 1987). Cells were harvested by centrifugation at 4300 × g for 10 min at 4°C and the pellets were washed once with 10 ml ice-cold TE buffer (50 mM Tris-HC1, 20 mM EDTA, pH 8.0). The suspensions were centrifuged as before and the pellets suspended in 8.5 ml TE. The suspensions were placed on ice and 0.5 ml 20% (w:v) N-lauroylsarcosine (Sigma L-5125) and 1.0 ml protease (pronase E, Sigma P-6911) solution (5 mg ml^{-1}) were added, followed by mixing and incubation at 50°C for 1 h. The protease solution had been predigested by incubation at 37°C for 90 min, then stored at –20°C. Pure total DNA was isolated from the lysates by standard methods (Sambrook et al., 1989). For Southern blot analysis, total DNAs were digested with *Bam*HI and subjected to electrophoresis in 0.7% (w:v) agarose (Sigma, Type V) in 1× TAE at 20V overnight. The DNA was transferred to a nylon membrane and hybridized (Sambrook et al., 1989) with ^{32}P-dCTP-labeled (Amersham random prime labeling kit and radionuclide, 3000 Ci $mmol^{-1}$) probe DNA, a *Bam*HI fragment of the ribosomal RNA gene probe pBJ142.

3. Results and Discussion

Keyser et al. (1982) reported, without specifying the cultivar used, that fast-growing soybean rhizobia from Chinese soil did not nodulate *Medicago sativa*. As judged by plant top dry weight and N content as well as nodule number under nil combined nitrogen growth conditions, the *Rhizobium fredii* type strain USDA 205 effectively and efficiently nodulated *M. sativa* cv. 'Giza 4' (Table 1), evidently forming only a slightly less efficient symbiosis with this cultivar than did *R. meliloti* strain ARC 1, which we previously described as an inoculant-quality, alfalfa-nodulating strain (Kuykendall et al., 1994). Nevertheless plants nodulated by strain 205 were tall, green and healthy compared to the short, yellow and sick plants that had not been inoculated. We then asked the questions, "Is the anomalous nodulation of alfalfa by *R. fredii* unique to this strain and is it cultivar-specific?"

Next, the symbiotic competence of twenty-two *R. fredii* strains and *B. japonicum* strain I-110ARS were evaluated with *M. sativa* cv. 'ARC', a standard improved U.S.-bred cultivar, in order to rule out the possibility that 'Giza 4' and strain 205 were both atypical genotypes in terms of their host/

Table 1. Nodulation of *Medicago sativa* cv. 'Giza 4' by *R. fredii* strain USDA 205 and *R. meliloti* strain ARC 1.

Strain	Nodule number	Shoot dry wt. (mg)	Plant N content %	mass (mg)
Uninoculated control	0	100	0.74	0.73
R. meliloti ARC 1	159	500	2.83	14.15
R. fredii USDA 205	58	420	2.21	9.28

Values are per 10 plants.

microsymbiont interactions. Three *R. meliloti* strains, recognized alfalfa microsymbionts, were included for comparison. Eight *R. fredii* strains nodulated *M. sativa* cv. 'ARC'. As evidenced by plant height and color, USDA strains 201, 208 and 214 effectively nodulated and efficiently fixed nitrogen with 'ARC' alfalfa, whereas USDA strains 196, 201, 205, 207, 209, and 217 were relatively poor microsymbionts; top dry weight data clearly indicate the relatively high symbiotic competence of the former over the latter (Table 2). Most strains nodulating alfalfa were of the original set described by Keyser et al. (1982), except for strains 207 and 209. Several *R. fredii* strains had higher nitrogenase activities on 'ARC' alfalfa than did *R. meliloti* strains USDA 1969 and USDA 1954. Only *R. fredii* strains USDA 201, USDA 208 and USDA 214 produced tall, green and healthy plants and exhibited high acetylene reduction activities comparable to those of the most effective *R. meliloti* strains with the 'ARC' cultivar (Table 2). Nodule reisolates, obtained from root nodules of 'ARC' alfalfa plants inoculated with *R. fredii*, were examined for their plasmid DNA content. The number of plasmids and their sizes (plasmid profile) of each group of nodule reisolates for USDA strains 205, 201, 208, 214, and 217 was identical to that of the respective original *R. fredii* strain as previously described (Hashem et al., 1996). Each had a unique plasmid profile that was different from those of the *R. meliloti* strains studied. Nodule reisolates of USDA strains 1936, 201, 208 and 214 were used to inoculate *M. sativa* cv. 'ARC' and *Glycine max* cv. 'Peking'. *R. meliloti* strain USDA 1936 was an effective and efficient symbiont with 'ARC' alfalfa, but it did not nodulate soybean. However, *R. fredii* strains 201, 208, and 214 were symbiotically competent with both of these agronomically important legumes (Table 3). RFLP analysis of chromosomal genes (Fig. 1) showed that the nodule reisolates of *R. fredii* were identical to the culture collection strains which had served as inocula for alfalfa plants. Alfalfa-nodulating *Rhizobium* strains of

Table 2. Symbiotic competence among *R. fredii* strains that nodulate *M. sativa* cv. 'ARC'. Values±standard deviation/5 plants

Strain	Nodule number	Shoot dry wt. (mg)	N_2-ase activity (μmole h^{-1})
Uninoculated control	0 ± 0	62 ± 6	0 ± 0
R. meliloti strain			
USDA 1936	85 ± 8	543 ± 73	48.0 ± 7.6
USDA 1954	58 ± 11	227 ± 55	29.3 ± 3.4
USDA 1969	60 ± 17	204 ± 21	16.7 ± 2.3
R. fredii strains			
USDA 196	15 ± 4	123 ± 18	10.0 ± 1.8
USDA 201	60 ± 14	203 ± 10	29.1 ± 2.1
USDA 205	27 ± 3	116 ± 28	3.1 ± 1.0
USDA 207	25 ± 5	78 ± 12	11.0 ± 1.5
USDA 208	72 ± 16	300 ± 14	39.1 ± 4.6
USDA 209	57 ± 15	233 ± 14	6.5 ± 1.3
USDA 214	65 ± 20	355 ± 34	37.0 ± 6.1
USDA 217	19 ± 4	87 ± 6	7.0 ± 1.2

Table 3. Symbiotic nitrogen fixation abilities of nodule reisolates with soybean cv. 'Peking' and 'ARC' alfalfa.

Strain	'ARC'[a]		'Peking'[b]		
	Shoot dry wt. (mg)	N_2-ase activity (μmole h^{-1})	Nodule number	Shoot dry wt. (mg)	N_2-ase activity (μmole h^{-1})
Uninoculated control	107 ± 13	0 ± 0	0 ± 0	697 ± 35	0 ± 0
USDA 1936	237 ± 27	12.6 ± 3.6	0 ± 0	717 ± 25	0 ± 0
USDA 201	186 ± 14	6.4 ± 1.9	21 ± 1	1630 ± 45	1.4 ± 0.4
USDA 208	228 ± 28	11.3 ± 1.5	53 ± 3	2472 ±132	11.7 ± 1.9
USDA 214	196 ± 11	5.6 ± 1.4	47 ± 8	2343 ±211	9.8 ± 1.5

[a]Values are means of 10 plants ± the standard deviation. [b]Values are means of 4 plants ± the standard deviation.

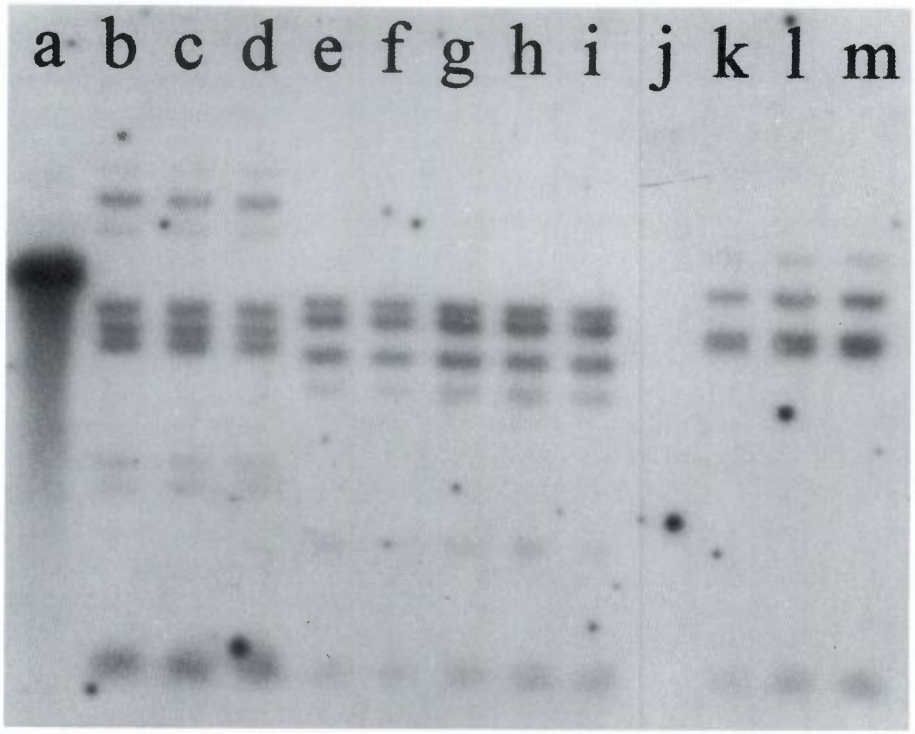

Figure 1. Autoradiograph showing bands of hybridization of pBJ142 DNA with *Bam*HI-restricted total DNAs of some of the *Rhizobium* strains examined in this study. Lane a, pBJ142 positive control; lane b, USDA 205; lanes c and d, reisolates of USDA 205; lane e, USDA 208; lanes f and g, reisolates of USDA 208; lane h, USDA 214; lane i, reisolate of USDA 214; lane k, USDA 1936; lanes l and m, reisolates of USDA 1936.

agronomic importance in Egypt fell into two genotypically distinct RFLP groups (Kuykendall et al., 1994) that may correspond to the two distinct groups of *R. meliloti* shown by isozyme analysis (Eardly et al., 1990), but this has not yet been tested.

Nod factors produced by *R. fredii* USDA 257 are not sulfated but rather have 2-*O*-methylfucose at the *O*-6 of the reducing N-acetylglucosaminosl residue (Bec-Ferté et al., 1994) whereas the *R. meliloti* Nod factor is sulfated at this position (Lerouge et al., 1990). This difference in substitution is currently thought to be a key determinant in host specificity. Our results clearly show that *R. fredii* and *R. meliloti* are more similar than earlier presumed in that they share a symbiotic affinity with *Medicago sativa*. Thus, our discovery

that strains of *Rhizobium fredii* are symbiotically competent with both alfalfa and soybean has significant implications for research into the biochemical basis of host specificity. Extrapolating conclusions about a species based only on one strain, such as *Rhizobium fredii* 257, is precarious; we propose that more strains be characterized as to the Nod factors produced. One important new question raised by this research is, "Do the alfalfa-nodulating *R. fredii* strains produce a Nod factor similar to that produced by *R. meliloti*?"

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