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Symbiotic Competence of *Sinorhizobium fredii* on Twenty Alfalfa Cultivars of Diverse Dormancy

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Abstract

Effective nodulation and efficient symbiotic biological nitrogen fixation with two alfalfa cultivars, 'ARC' and 'Giza 4' by some strains of the fast-growing soybean microsymbiont species *Sinorhizobium fredii* was recently observed for the first time. However, the host specificity and symbiotic competence of these strains on a range of genetically diverse alfalfa cultivars had not yet been investigated. Therefore, in this study, twenty genetically distinct alfalfa cultivars that differ in origin and fall dormancy, from fall non-dormant to completely dormant, were inoculated with each of four *S. fredii* strains or inoculated with *S. meliloti* strain USDA 1936, grown in the growth-chamber or in the greenhouse for six weeks and then examined for growth vigor, nodulation and nitrogenase activities. Whereas *S. fredii* strains USDA 205 and USDA 208 effectively nodulated all of the alfalfa cultivars, strains USDA 201 and USDA 214 nodulated only two and six cultivars, respectively. The latter strains nodulated some of the semi-dormant and dormant alfalfa genotypes, but failed to nodulate any of the non-dormant genotypes. In many cases, strains USDA 205 and USDA 208 were as symbiotically competent with the alfalfa cultivars tested as was the alfalfa microsymbiont *S. meliloti* strain USDA 1936. Therefore, this study clearly shows that nodulation of alfalfa by *S. fredii* type strain USDA 205 and strain USDA 208 is not cultivar specific.

Keywords: Biological nitrogen fixation, endophyte, host specificity, *Medicago sativa*, microsymbionts, *Rhizobium meliloti*, symbiosis

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1. Introduction

Soybean, *Glycine max* (L.) Merr., and alfalfa, *Medicago sativa* L., are major world grain and forage legume crops, respectively. The relationship between these crops and their microsymbionts was thought to be highly specific, with alfalfa being nodulated by *S. meliloti*, and soybean nodulated by microsymbionts from two genera: slow-growing *Bradyrhizobium japonicum, B. elkanii, B. liaoningense,* and fast-growing *Sinorhizobium fredii*. Recently, we reported (Hashem et al., 1997) that strains of the fast-growing soybean-nodulating bacteria, *S. fredii*, effectively nodulate alfalfa and the soybean cv. 'Peking'. Furthermore, *S. fredii* strain USDA 257 (Krishnan and Pueppke, 1994) and *Sinorhizobium* sp. strain NGR234 (Dénarié, 1993) had been shown to have broad host ranges since these strains nodulate more than 60 different legume genera and the non-legume *Parasponia*. In addition, *Rhizobium* sp. (*Leucaena*) strain DS 65 nodulated several small- and large-seeded legume plant species including *Leucaena culensi, L. retusa, L. divusiflora, Glycine max, Vigna sinensis* and *Phaseolus vulgaris* but this strain did not nodulate *Medicago sativa* (Swelim et al., 1997). *S. meliloti* has a narrow, restricted host range with species of *Medicago, Melilotus* and *Trigonella* (Jordan, 1984), though Gao and Yang (1995) recently reported that a Chinese field isolate of *S. meliloti* effectively nodulated soybean in China.

S. fredii has a close phylogenetic relationship with *S. meliloti* and they are currently both assigned to the new genus *Sinorhizobium* (Chen et al., 1988; De Lajudie et al., 1994; Martinez-Romero and Caballero-Mellado, 1996). Close phylogenetic relationship is indicated by 16S rRNA sequence analysis. The nucleotide sequence of a particular 260-bp segment of the 16S rRNA genes of representative strains was identical (Javis et al., 1992). They overlap serologically (Sadowsky et al., 1987), and both bacterial species carry megaplasmids as large or larger than 1,000 MDa (Hashem and Kuykendall, 1994; Hashem et al., 1996). Moreover, Hashem et al. (1996) recently reported that some *S. fredii* phages also infected *S. meliloti*.

Effective nodulation of both the Egyptian alfalfa cultivar 'Giza 4' and the USDA cultivar 'ARC' by several *S. fredii* strains was recently reported (Hashem et al., 1997). Only two alfalfa varieties that differ in their dormancy were previously examined. The degree of dormancy determines the adaptability to winter survival and thus the areas of adaptation. Alfalfa cultivars with greater dormancy are more winter hardy and thus adapted to regions of severe winter while the non dormant type can grow continuously through the winter and are adapted to regions that are frost free or suffer only minimal frost. Therefore, the main objectives of this study were as follows: 1. To examine twenty genetically different cultivars of *M. sativa* that differ in

their dormancy (non-dormant through dormant) classes and origin for nodulation and biological nitrogen fixation with the fast-growing soybean-nodulating species, *S. fredii*. 2. To determine whether or not alfalfa symbioses formed with *S. fredii* are cultivar specific.

2. Materials and Methods

Sinorhizobium strains and alfalfa genotypes

Sinorhizobium strains were obtained from the USDA, ARS *Rhizobium* Germplasm Resource Center, Beltsville, Maryland, USA. *S. fredii* strains USDA 201, USDA 205, USDA 208, and USDA 214 were reported to effectively nodulate both 'ARC' alfalfa and 'Williams' soybean (Hashem et al., 1997). *S. meliloti* strain USDA 1936 was used as a positive reference strain. *S. meliloti* strain USDA 1936 and the *S. fredii* strains have been characterized for plasmid content (Hashem and Kuykendall, 1994), for phage sensitivity (Hashem et al., 1997), and for symbiotic competence with both alfalfa and soybean (Hashem et al., 1997; Kuykendall et al., 1994). *Sinorhizobium* 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 inoculant. Strains were maintained by storage in 50% glycerol at –70°C.

The twenty different cultivars of *Medicago sativa* that were used in this study are listed (Table 1). *M. sativa* L. cv. 'ARC', a USDA-developed cultivar with multiple pest resistance (Devine et al., 1975), was a reference host plant. Seeds of *M. sativa* were from the Soybean and Alfalfa Research Laboratory, Plant Sciences Institute, Agricultural Research Services, United States Department of Agriculture, Beltsville, MD, USA.

The *Medicago sativa* cultivars evaluated for symbiosis with *S. fredii* were selected so as to represent all of the fall dormancy classes as well as the dormancy variability for North American alfalfa cultivars (Table 1). Dormancy classes are determined by scoring plants for regrowth 21 to 25 days after clipping the plants down to their crowns in the fall of the year. Dormancy classes are assigned based on plant regrowth height on a scale of 1 to 9, with a rating of 9 = >40 cm, 8 = >35 – <40 cm, 7 = >30 – <35 cm, 6 = >25 – <30 cm, 5 = >20 – <25, 4 = >15 – <20, 3 = >10 – <15, 2 = >5 – <10, and 1 = 0–5 cm. The amount of regrowth of alfalfa in the fall is correlated with fall dormancy, thus a dormancy class of 1 is very fall dormant and 9 is non-dormant (Barnes et al., 1978; Barnes et al., 1995). Each of the fall dormancy classes was represented by a fall dormancy check variety as prescribed in the Standard Tests to Characterize Alfalfa Cultivars (Barnes et al., 1995). Both public and

Table 1. Origin and dormancy classes of the alfalfa cultivars used in this study.

Dormancy rating	Alfalfa cultivars	Origin/source
1	Beaver*	Saskatchewan, Canada
2	Vernal*	Wisconsin, USA
2	Alfagraze	American Alfalfa
2	Pacesetter	Research Seeds
3	Ranger*	Nebraska and North Dakota , USA
3	5246	Pioneer Hi-Bred International
3	Oneida	New York, USA
4	ARC	Maryland, USA
4	Saranac*	New York, USA
4	Wl322 HQ	WL Research
4	Legand	Cenex/Land O'Lakes
4	Cimmaron	Great Plains
5	DuPuits*	France
6	Lahonton	Nevada, USA
6	ABI700*	American Alfalfa
7	Mesilla*	New Mexico, USA
8	Moapa 69*	Nevada, USA
8	Mesa Sirsa	PGI/MBS
9	CUF 101*	California, USA
9	Florida	Pioneer Hi-Bred International

Dormancy class: 1 = Very dormant; 9 = Non-dormant. *Standard Check Variety for Dormancy.

proprietary cultivars were evaluated (Table 1) as was the cultivar 'ARC' which was previously observed to be nodulated with both *S. meliloti* and *S. fredii* (Hashem et al., 1997).

Host-microbe interactions and the effectiveness tests

Seed of each of the *M. sativa* L. cultivars were scarified and then were surface-sterilized by immersion in 95% ethanol for 10 s and then soaked in a 3% (v/v) sodium hypochlorite solution for 3 min and then rinsed several times with sterilized distilled water (Somasegaran and Hoben, 1994). Seed were then planted in 50 × 5 cm glass tubes containing nitrogen-free soft agar medium (Abdel-Gaffar and Jensen, 1966), and inoculated with a single strain of either *S. meliloti* strain USDA 1936 or *S. fredii* strains USDA 201, USDA 205, USDA 208, or USDA 214. Tubes containing uninoculated seed served as controls. Growth tubes were arranged randomly in a complete randomized design with

four replications of each treatment. The experiment was repeated three times. At six weeks following inoculation, growth and nodulation of plants were evaluated, and nitrogenase activities in the growth tubes were estimated by the acetylene reduction assay.

Greenhouse tests

Additional effectiveness tests were conducted in the greenhouse to confirm the findings of previous tests, and to examine the ability of *S. fredii* strains to nodulate and to fix nitrogen with *M. sativa*. Surface-sterilized seeds of *M. sativa* were planted in autoclaved Leonard jars (Leonard, 1943) containing sterile vermiculite and N-free plant nutrient solution (Norris, 1964). Each jar received three milliliters of an early log-phase broth culture of each strain. There were four replicate jars in each treatment. Four jars were left uninoculated as controls. Jars were arranged in the greenhouse in a completely randomized design. After six weeks, plants were harvested, and nodulation, plant growth and nitrogenase activities were determined.

Nitrogenase activity analysis

Five plants from each Leonard jar were carefully uprooted six weeks after planting. Plant tops were cut and each root system was gently separated from the soil and placed in a reaction jar fitted with a rubber septum. For plants grown in growth-test tubes in the growth chamber, the cotton plugs were removed from the tubes and replaced with rubber septums. The air in each jar or growth tube was amended with 10% acetylene and the jars or growth tubes were incubated at room temperature (25°C) for 30 minutes. Three samples of gas from each jar were analyzed for ethylene production by gas chromatography (Kummer and Kuykendall, 1989).

Statistical analysis

Data were subjected to an analysis of variance using the SAS Series in Statistical Applications: SAS System for Linear Models (Littell et al., 1993). When significant effects were found (P 0.05), means were separated using the least significant difference, LSD(0.05).

3. Results and Discussion

The report of Hashem et al. (1997) that some strains of *S. fredii* nodulate and effectively fix nitrogen with two cultivars of alfalfa, 'ARC' and 'Giza 4',

Figure 1. Enhanced plant growth of 'ARC' alfalfa by the inoculation with single strain inocula of *S. fredii* strains USDA 201, USDA 208 and USDA 214 or *S. meliloti* strain USDA 1936.

clearly contradicted the report of Keyser et al. (1982), that concluded that fast-growing soybean rhizobia from Chinese soils do not nodulate *M. sativa*. This discrepancy would be explained if nodulation was cultivar-specific. After all, Keyser et al. (1982) used only one cultivar and did not specify the alfalfa cultivar tested. We examined twenty genetically different cultivars of alfalfa that represented all of the fall dormancy classes to determine whether nodulation of alfalfa by *S. fredii* has cultivar specificity for nodulation and symbiotic nitrogen fixation with single strain inocula of four *S. fredii* strains. *S. meliloti* strain USDA 1936 and *B. japonicum* strain I-110 (Kuykendall and Elkan, 1976) served as positive and negative reference strains, respectively, on all twenty alfalfa cultivars.

In Leonard jar tests in the greenhouse, *S. meliloti* USDA 1936 and *S. fredii* strains USDA 201, USDA 208 and USDA 214 effectively nodulated 'ARC' alfalfa, efficiently fixed nitrogen, and thus clearly conferred nitrogen sufficiency to plants grown in nitrogen-limited medium (Fig. 1), whereas

Figure 2. Enhanced plant growth (A) and nodulation (B) of 'ARC' alfalfa by the inoculation with single strain inocula of *S. fredii* strain USDA 214 or *S. meliloti* strain USDA 1936.

uninoculated plants were stunted and yellow. The extent of nodulation on the 'ARC' alfalfa root systems was obviously comparable between *S. fredii* strain USDA 214 and *S. meliloti* strain USDA 1936 (Fig. 2).

In growth-chamber tests, as judged by plant height, color and nitrogenase activities, *S. fredii* strains USDA 201, USDA 205, USDA 208 and USDA 214 fixed nitrogen with two or more of the *M. sativa* cultivars examined (Tables 2 and 3). These four strains, however, varied considerably in their ineffectiveness and effectiveness with the various alfalfa cultivars (Figs. 3–6). Strains USDA 205 and USDA 208 produced more vigorous alfalfa cv. 'Alfagraze' plants than did any of the other treatments. Relative vigor of these plants was correlated with significant differences in nitrogenase activities (Fig. 3, Table 3). Alfalfa cultivar 'Ranger' was benefited most by nodulation by strains USDA 205 and USDA 1936, and this difference was correlated with the rate of acetylene reduction (Fig. 4, Table 3). 'DuPuits' was effectively nodulated by strains USDA 205, USDA 208, and USDA 1936,

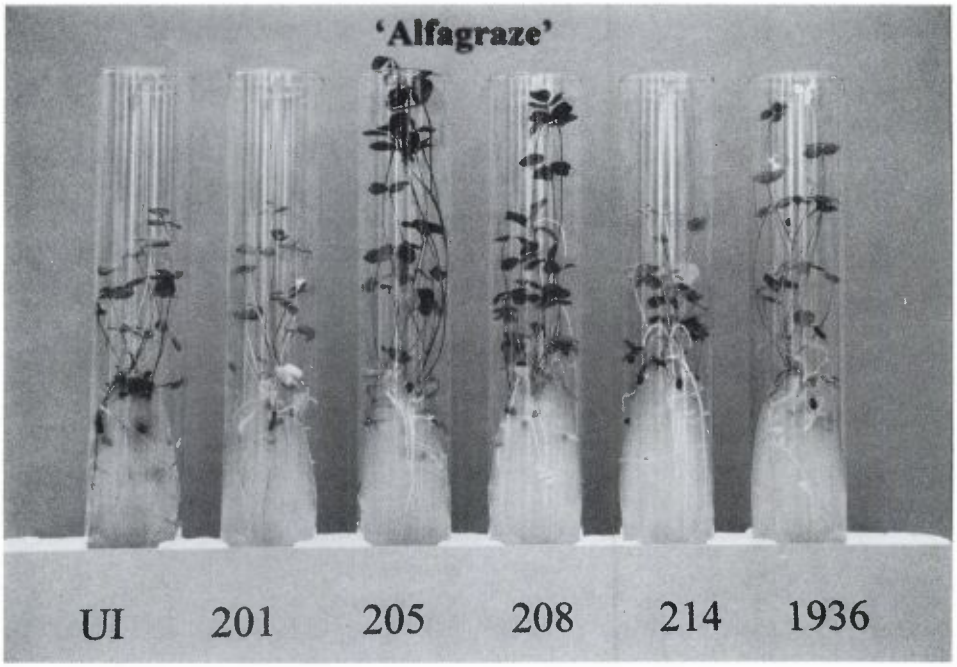

Figure 3. Host specificity of *S. fredii* strains USDA 201, USDA 205, USDA 208, USDA 214 and *R. meliloti* strain USDA 1936 on 'Alfagraze' alfalfa that was grown in a growth chamber.

and this also was correlated with the nitrogenase activities (Fig. 5, Table 3). With alfalfa cultivar 'ABI 700', strain USDA 205 was the superlative microsymbiont as evidenced by both plant height and nitrogenase activities (Fig. 6, Table 3). On the other hand, strain USDA 201 nodulated only two alfalfa cultivars, 'ARC' and 'Oneida', and strain USDA 214 nodulated only six alfalfa cultivars. In both cases, some semi dormant and dormant class genotypes were nodulated but none of the non-dormant genotypes were nodulated by these two strains under the conditions used. Therefore, strains USDA 201 and USDA 214 nodulated only 10% and 30%, respectively, of the alfalfa cultivars examined. However, all of the alfalfa cultivars examined were nodulated by *S. fredii* strains USDA 205 and USDA 208 (Table 2). Most plants nodulated by strains USDA 205 and USDA 208 were tall, green and healthy compared to the relatively short, yellow uninoculated plants (Table 2, Figs. 2–6). Therefore, we conclude that nodulation of alfalfa and

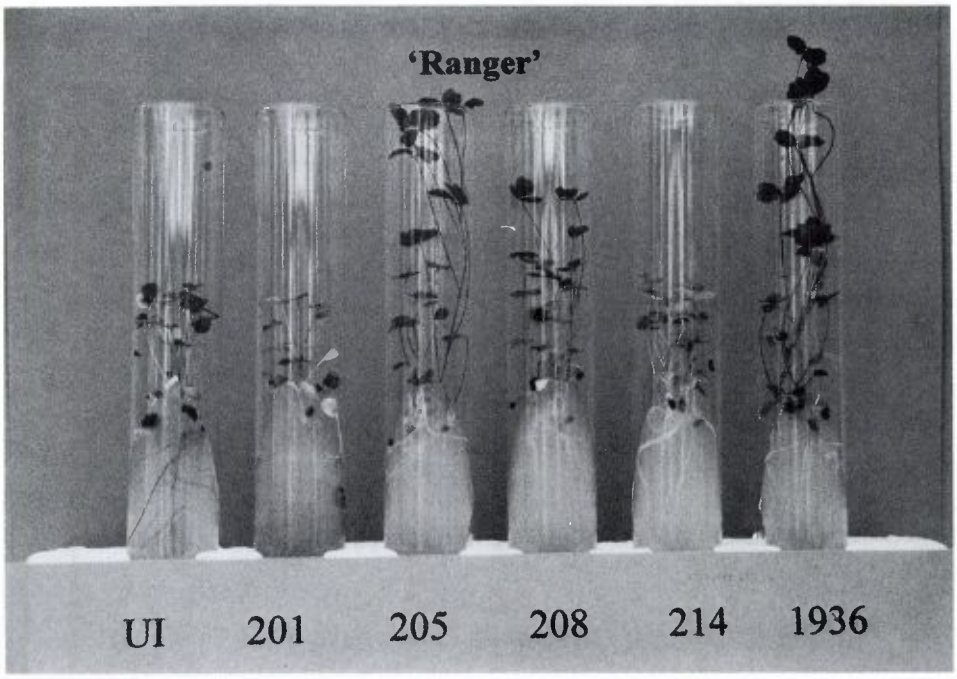

Figure 4. Host specificity of *S. fredii* strains USDA 201, USDA 205, USDA 208, USDA 214 and *R. meliloti* strain USDA 1936 on 'Ranger' alfalfa that was grown in a growth chamber.

biological nitrogen fixation by *S. fredii* strains USDA 205 and USDA 208 are clearly not cultivar-specific. This may be due to the low genetic diversity among alfalfa cultivars (Kidwell et al., 1994). These investigators used RFLP analyses to establish that most alfalfa germplasm sources are not very genetically diverse.

The symbiotic effectiveness of *S. fredii* strains, three *R. meliloti* strains for comparison and *B. japonicum* strain I-110ARS as a negative reference strain, were previously evaluated (Hashem et al., 1997) with *M. sativa* cv. 'ARC', a standard cultivar, in order to rule out the possibility that 'Giza 4', the Egyptian alfalfa cultivar initially observed to be nodulated by strain USDA 205, was atypical. In that previous study, eight *S. fredii* strains were found to nodulate *M. sativa* cv. 'ARC'. As evidenced by plant height and color, plant top dry weights and acetylene reduction assays, *S. fredii* strains USDA 208 and USDA 214 in particular effectively nodulated and efficiently fixed nitrogen

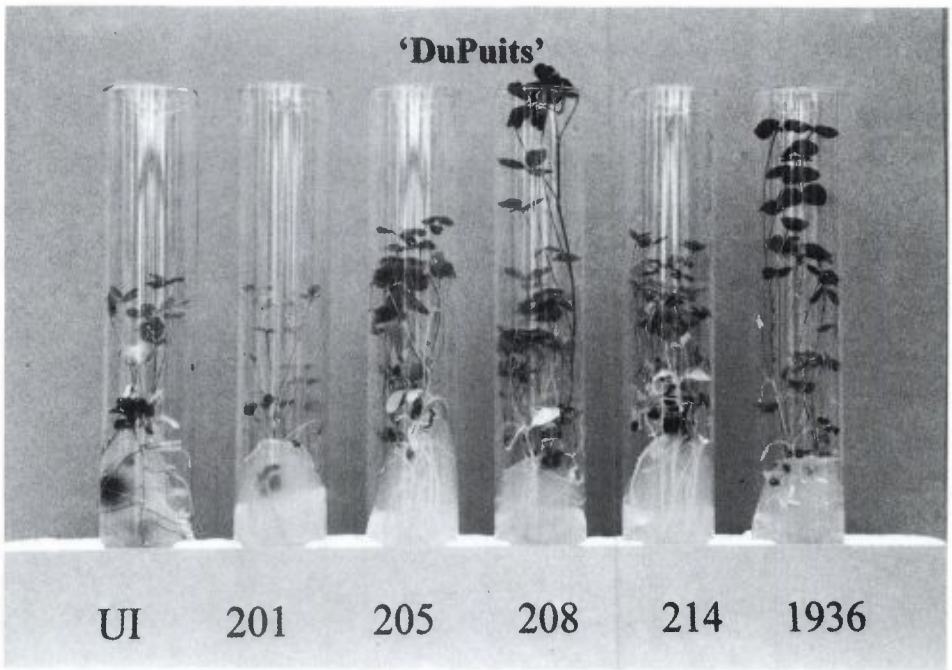

Figure 5. Host specificity of *S. fredii* strains USDA 201, USDA 205, USDA 208, USDA 214 and *R. meliloti* strain USDA 1936 on 'DuPuits' alfalfa that was grown in a growth chamber.

with 'ARC' alfalfa, whereas strains USDA 196, USDA 201, USDA 205, USDA 207, USDA 209, and USDA 217 formed nodules that were relatively inefficient at fixing nitrogen.

The present study provides new and statistically significant data showing that, on most alfalfa cultivars examined, the two *S. fredii* strains, USDA 205 and USDA 208, formed symbioses that had nitrogenase activities equal to or higher than those exhibited by *S. meliloti* strain USDA 1936 (Table 3). In our earlier study (Hashem et al., 1997), *S. fredii* strains USDA 208 and USDA 214 were symbiotically competent with alfalfa. This present study also shows that *S. fredii* strains USDA 205 and USDA 208 both nodulated and formed efficient nitrogen-fixing symbioses with most alfalfa cultivars tested. Two other *S. fredii* strains either did not nodulate most of the cultivars tested or nodulated and fixed nitrogen relatively poorly with very few of the alfalfa cultivars. Strains USDA 201 and USDA 214 exhibited relatively low acetylene reduction activities with the alfalfa cultivars that they nodulated.

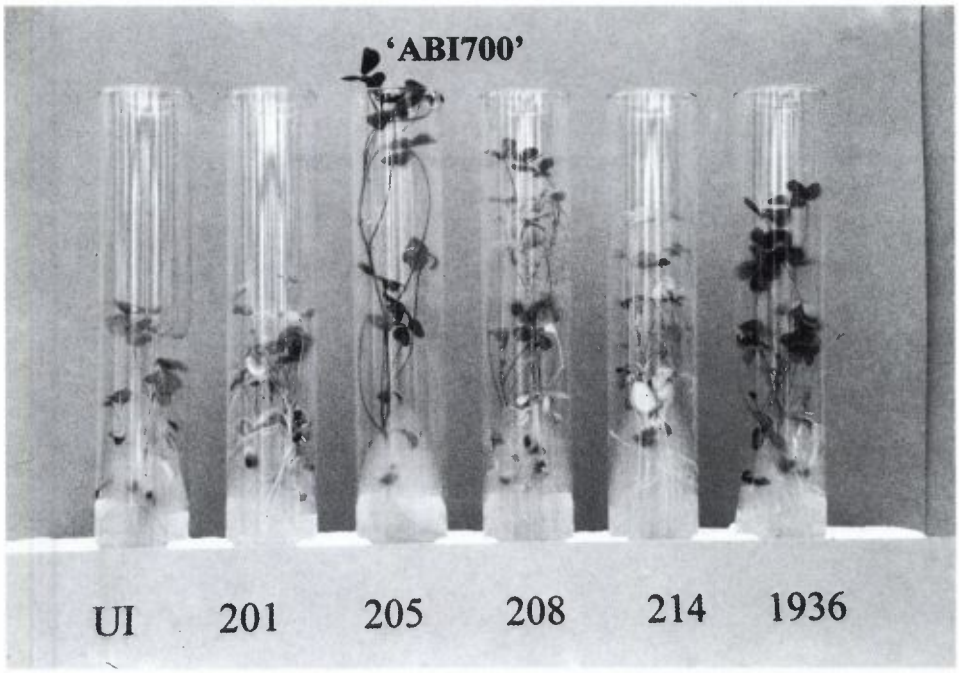

Figure 6. Host specificity of *S. fredii* strains USDA 201, USDA 205, USDA 208, USDA 214 and *R. meliloti* strain USDA 1936 on 'AB1700' alfalfa that was grown in a growth chamber.

The present investigation documents the diversity of *S. fredii* strains in nodulating diverse genotypes of alfalfa. This diversity among the strains is also documented in other studies. Hashem et al. (1997) reported that strains USDA 201, USDA 205, USDA 208 and USDA 214 varied significantly on their symbiotic competence with both alfalfa cv. 'ARC' and soybean cv. 'Peking', with strain USDA 208 being the most competent with these plant species. Furthermore, these strains varied in their plasmid DNA content and in their susceptibility to *S. fredii* phages since strains USDA 201, USDA 205, USDA 208 and USDA 214 carry 3, 4, 2 and 2 plasmid, and they are susceptible to 0, 2, 4 and 0 rhizobiophages, respectively (Hashem et al., 1996).

The mechanisms controlling host specificity in the legume/*Rhizobium* symbioses are poorly understood. For example, the present study shows clearly that fast-growing soybean nodulating microsymbionts from China are also good nitrogen-fixing symbionts for alfalfa. Furthermore, these legume-nodulating bacteria are not as host-specific as earlier believed. The discovery that the

Table 2. Nodulation and plant vigor of diverse alfalfa genotypes inoculated with single strain inocula of *S. fredii* strains USDA 201, USDA 205, USDA 208 and USDA 214, *S. melilotii* strain USDA 1936, or uninoculated.

Alfalfa cultivars	*Sinorhizobium* strains									
	Control		USDA 1936		USDA 201		USDA 205		USDA 208		USDA 214	
	Nod[1]	Vigor[2]	Nod	Vigor	Nod	Vigor	Nod	Vigor	Nod	Vigor	Nod	Vigor
Beaver	– [1]	2 [2]	++	4	–	1	+	5	+	4	+	2
Vernal	–	2	++	4	–	2	+	4	++	4	–	1
Alfagraze	–	2	++	4	–	2	++	5	++	5	+	2
Pacestter	–	2	++	4	–	2	+	4	++	4	–	2
Ranger	–	1	++	5	–	1	+	5	++	4	–	1
5246	–	2	++	3	–	2	+	4	+	4	–	2
Oneida	–	2	++	4	+	3	+	4	+	5	–	2
ARC	–	1	++	5	+	2	++	5	+	4	+	2
Saranac	–	2	++	4	–	2	+	5	++	3	+	1
WL 322 HQ	–	2	++	3	–	2	+	4	++	4	+	2
Legand	–	2	++	4	–	2	+	3	++	4	+	2
Cimmaron	–	2	++	5	–	2	+	3	++	4	–	2
DuPuits	–	1	++	5	–	1	+	4	++	5	–	3
Lahonton	–	2	++	4	–	1	+	5	++	5	–	3
AB1700	–	2	++	4	–	2	++	5	++	4	–	3
Mesilla	–	2	++	5	–	2	+	4	+	3	–	3
Moapa 69	–	2	++	5	–	2	+	3	++	5	–	2
Mesa Sirsa	–	2	++	5	–	2	+	3	+	5	–	3
CUF 101	–	1	++	5	–	2	+	4	+	4	–	3
Florida 77	–	2	++	3	–	2	+	2	++	4	–	2

[1]Nodulation score: – = No nodules, + = between one and three nodules, and ++ = four nodules or more. [2]Plant growth, height, color and vigor were scored in a scale of 1 to 5, with 1 being the least growth, stunt, and yellow plants, and 5 for plants that were the tallest and most green.

Table 3. Nitrogenase activities of Alfalfa genotypes inoculated with single strain inocula of *S. fredii* strains USDA 201, USDA 205, USDA 208, USDA 214, or *S. meliloti* strain USDA 1936.

Alfalfa cultivars	Control	*Sinorhizobium* strains					LSD(0.05)
		USDA 1936	USDA 201	USDA 205	USDA 208	USDA 214	
		(μmole C_2H_4/growth tube/h)					
Beaver	0	25.7[1]	0	16.4	26.9	7.1	3.0
Vernal	0	23.4	0	23.9	23.9	0	5.3
Alfagraze	0	12.2	0	29.4	22.1	7.2	3.3
Pacestter	0	13.9	0	22.0	12.7	0	1.9
Ranger	0	23.4	0	27.8	20.8	0	5.0
5246	0	8.9	0	26.8	21.6	0	7.8
Oneida	0	15.4	11.9	17.9	26.3	0	3.6
ARC	0	28.4	6.9	14.0	28.2	13.7	7.8
Saranac	0	21.8	0	24.9	5.4	5.3	3.6
WL 322 HQ	0	6.9	0	26.3	16.7	8.1	3.0
Legand	0	14.2	0	21.0	15.4	12.3	4.0
Cimmaron	0	21.0	0	20.2	14.5	0	6.1
DuPuits	0	24.9	0	23.0	26.1	0	6.2
Lahonton	0	15.9	0	23.3	23.4	0	4.4
AB 1700	0	15.1	0	32.9	17.2	0	6.5
Mesilla	0	18.5	0	32.6	13.2	0	5.6
Moapa 69	0	24.3	0	7.6	27.1	0	1.9
Mesa Sirsa	0	28.9	0	15.9	22.7	0	3.8
CUF 101	0	20.6	0	19.9	16.9	0	3.6
Florida 77	0	5.4	0	6.7	15.9	0	2.3
LSD(0.05)	0	6.1	0.5	7.4	4.3	0.9	

[1]Values represent the mean of four replications.

bacterial species *S. fredii* effectively fixes nitrogen symbiotically with both alfalfa and soybean may revolutionize theories concerning the role of certain cell-wall constituent-like pentasaccharide fatty acid signals called "Nod factors." Nod Factors produced by *R. fredii* USDA 257 are not sulfated but rather have 2-*O*-methyl fucose at the *O*-6 of the reducing N-acetylglucosaminose residue (Bec-Ferté et al., 1994) whereas the *S. 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 (Pueppke and Broughton, 1999). Our results clearly show that *S. fredii* shares

with *S. meliloti* a symbiotic affinity with *M. sativa*. Thus, the finding that *S. fredii* strains nodulate and fix nitrogen with diverse alfalfa cultivars shows that these phenomena are not cultivar-specific and thus our finding clearly indicates the need for more host specificity and Nod Factor research.

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