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Characterization of Rhizobia from Ineffective Alfalfa Nodules: Ability to Nodulate Bean Plants [*Phaseolus vulgaris* (L.) Savi.]†

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This study was initiated to characterize *Rhizobium* isolates obtained from root nodules of ineffectively nodulated, field-grown alfalfa (*Medicago sativa* L.) plants. The purpose was to determine if these isolates possessed characteristics which would explain either their ineffectiveness in N_2 fixation or their apparent ability to tolerate the moderately acid soil conditions from which they originated. Isolates were characterized by analysis of growth rate, 39°C tolerance, acid production on conventional media, and symbiotic performance. All isolates were ineffective in N_2 fixation on alfalfa, and they contained one or more anomalous characteristics. These included either slow growth rate, lack of 39°C tolerance, or lack of acid production on conventional media. Infectiveness tests on a broad range of legumes revealed that the isolates formed root nodules on *M. sativa*, *Medicago lupulina* L., and *Phaseolus vulgaris* (L.) Savi. (common bean). These results provide evidence that, in some situations, ineffective nodulation of *M. sativa* in the field may be due to the presence of promiscuous, native *Rhizobium* species.

In describing symbiotic N_2 fixation potential between bacteria of the genus *Rhizobium* and their respective plant hosts, the terms effective and ineffective are frequently used. Effective symbiotic associations supply sufficient amounts of reduced N to the plant host for plant development; ineffective associations do not reduce N_2. Ineffective symbiotic relationships can be caused by either the microsymbiont (29) or the macrosymbiont (36).

Environmental factors such as soil acidity often influence the effectiveness of symbiotic relationships. The microsymbiont which infects *Medicago sativa* L. (alfalfa) is particularly acid sensitive in either broth cultures (18) or soil (1, 25, 26, 30, 32, 33). Moderate acidity (pH 5.5) in nutrient solutions also has a negative effect on the rhizobial infection process in alfalfa (27). A negative correlation between soil pH and the presence of ineffective nodules on alfalfa in greenhouse studies also has been reported (5, 31).

Field and greenhouse observations, using soils from various locations in the northwestern United States (1, 4, 12, 39), Canada (8, 31), and Australia (5, 6, 7), have indicated that the persistence of ineffective *Rhizobium meliloti* in moderately acid soils is a common yet unexplained phenomenon (9). Recent studies have indicated that the host plant may play a role in the acid tolerance of *R. meliloti* (13, 24). These studies did not explain the persistence of some ineffective *R. meliloti* strains under moderately acid soil conditions in the absence of the host plant.

This investigation was initiated in response to results of a study which indicated that a field site having moderately acid soil conditions contained only ineffective *R. meliloti* (12). Since alfalfa had not been grown on these sites for at least 10 years, it was of interest to determine if members of the soil population of *R. meliloti* possessed other unusual traits which would help explain their persistence and ineffectiveness. It was noted that, when grown in pure cultures, many nodule isolates from noninoculated alfalfa formed unusually small colonies which grew slowly in comparison with strains of *R. meliloti*, which formed effective symbiotic relationships. An investigation was initiated to characterize this native population by traditional methods. The objective was to verify that the native *Rhizobium* species were indeed *R. meliloti* and to provide a possible explanation for their anomalous growth rate and ineffectiveness.

MATERIALS AND METHODS

Source of strains. Nodule isolates from *M. sativa* (cv. Apollo) were obtained in 1981 and 1982 from two field sites located at the Hyslop Crop Science field research facility, Corvallis, Oregon. Alfalfa had been grown at both sites previously; however, during the past 10 years the plots had been either fallow or planted in wheat. There are no reports of indigenous *Medicago* species endemic to the region; however, *Medicago lupulina* L. has been observed growing as an introduced turfgrass weed (W. S. McGuire, personal communication). The soil at the sites was a moderately acid (pH 5.5 to 5.7) silt loam (Aquultic Argixeroll), which was limed to pH 6.2 at 1 month before planting. Seeds for trap plants were surface sterilized by standard procedures (37) by using a brief ethanol rinse followed by a 3-min immersion in acidified 0.2% (wt/vol) $HgCl_2$. The seeds were then rinsed in sterile water, dried, and planted at four separate locations within each field site. We also germinated 10 seeds in sterile closed plant tubes (3.0 by 20 cm) containing 20 ml of modified Jensen seedling agar (4, 37) to assess the efficacy of seed sterilization. In 1982, detailed analysis of N_2 fixation activity, herbage N composition, and yield of the field-grown plants was also evaluated, and the results suggested that the native isolates were ineffective in N_2 fixation (12).

Native *R. meliloti* isolates to be used for effectiveness and infectiveness testing were obtained by removing 16 noninoculated plants from random locations within the field plots each year. Two nodules were randomly selected from each plant and were surface sterilized by standard procedures (37). Nodules were then rinsed, squashed, and streaked to purity on plates of yeast-extract mannitol (YEM) agar containing 0.0025% (wt/vol) Congo red dye and 0.002%

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TABLE 1. Comparison of cultural and symbiotic effectiveness characteristics of several native *M. sativa* nodule isolates with other strains of *Rhizobium* species

Source of isolate or strain	Identification code	Acid production[a]	Mean generation time (h)	Shoot dry wt (mg) (effectiveness test)[b]
1981 Field isolate	81	±	8.7 ± 0.9	4.5 ± 0.3
	82	±	8.2 ± 0.6	4.3 ± 0.4
	84	−	5.9 ± 0.7	5.1 ± 0.2
1982 Field isolate	160	−	9.9 ± 1.4	4.8 ± 0.6
	166	−	3.4 ± 0.1	5.2 ± 0.5
	172	±	7.7 ± 0.9	4.5 ± 0.2
	188	−	8.5 ± 0.2	4.9 ± 0.7
	191	−	3.7 ± 0.2	5.4 ± 0.3
Nitragin Co.	102F34 (*R. meliloti*)	+	1.5 ± 0.2	19.8 ± 0.7
Nitragin Co.	127K12b (*R. phaseoli*)	+	NM[c]	NM
Private collection[d]	USDA 110 (*Bradyrhizobium japonicum*)	−	5.4 ± 0.2	NM
NA[e]	KNO_3 control	NA	NA	19.9 ± 0.4
NA	Noninoculated control	NA	NA	5.4 ± 0.2

[a] +, Acid production; ±, slight acid production; −, no acid production on YEM agar.
[b] Effectiveness tests performed on Apollo alfalfa.
[c] NM, Not measured.
[d] Collection of H. J. Evans.
[e] NA, Not applicable.

(wt/vol) cycloheximide (37). The method of Bernaerts and Deley (3) was used to confirm that the isolates were not members of the genus *Agrobacterium*. Plates were incubated at 30 ± 1°C for 4 to 7 days. Single colonies were picked and restreaked three times before storage on YEM slants at 4°C. Slants of all strains were removed from storage and restreaked every 3 to 4 months. Effectiveness tests, using Apollo alfalfa grown in plant tubes, confirmed that all isolates were ineffective in N_2 fixation (12).

Acid production and 39°C tolerance of selected nodule isolates. All isolates were initially screened for acid production by streaking onto YEM agar plates (pH 7.0) containing bromothymol blue indicator (28, 37). Color change was evaluated at 2, 5, and 9 days. The eight isolates listed in Table 1 were then selected for further examination based on diverse nodule characteristics, apparent growth rates on agar media, and acid production on YEM medium. Acid production by members of this group was confirmed by measuring pH depression in 50-ml YEM broth cultures (pH 7.0 and 9.5) after 1 week of growth at 30°C. The initial pH of the broth was adjusted by adding alkali aseptically after autoclaving. Cultures were grown in 250-ml Erlenmeyer flasks on a rotary shaker at 200 rpm.

Temperature tolerance of the same isolates was evaluated by incubating duplicate YEM plates at 30 and 39°C, respectively, for 1 week. In all evaluations, commercial inoculant strains and noninoculated controls were included for comparative purposes.

Mean generation time. Generation time was determined for the eight representative native field isolates and two control strains (Table 1). Three replicate flasks for each isolate were inoculated with 10^7 cells, and growth was monitored turbidimetrically in sidearm flasks, using the cultural conditions previously described.

Effectiveness of native isolates on alfalfa cultivars. Symbiotic effectiveness of the selected nodule isolates was assessed on several cultivars of alfalfa, using the closed plant tube method. A 5 × 6 factorial treatment combination was used. Treatments consisted of five cultivars of alfalfa (Vernal, Saranac, Buffalo, Apollo, and DuPuits) and six representatives of the field isolates (strains 081, 082, 084, 166, 172, and 191). The five cultivars were selected based on several criteria, including adaptation to local conditions (Apollo), previous planting history on the field site (DuPuits), and diverse varietal origin (Buffalo, Saranac, and Vernal) (2). Six plants were used per treatment, and the experiment was conducted in a growth chamber with a cycle of 14 h of daylight and 10 h of darkness at 22 and 20°C, respectively. Fluorescent and incandescent lights in the chamber provided a photon flux density of 500 μmol m^{-2} s^{-1}. A reisolation of strain 191 was obtained from an Apollo alfalfa plant, and the reisolate was designated 191R.

Infectiveness of nodule isolates on diverse legume hosts. The host specificity of three of the *Rhizobium* isolates (strains 082, 171, and 191R) was evaluated by inoculating seven representative legume hosts of the commonly used cross-inoculation groups (Table 2). The experiment was conducted in the growth chamber by using a 3 × 7 factorial treatment combination with three replications in a completely randomized design. With the exception of black medic (*M. lupulina*), seeds were surface sterilized with a brief 95% ethanol rinse followed by a 3-min immersion in 2.0% (vol/vol) NaOCl. Black medic seeds, which were collected locally and were apparently hard seeded, required a 10-min immersion in concentrated HCl followed by several sterile-water rinses to improve germination. All seeds were transferred to sterile-water agar (1% wt/vol) for germination.

Germinated seedlings (one per tube) were grown in modified open plant tubes (37). Before planting, the plant tubes (3.0 by 20 cm) were filled with coarse, washed vermiculite and capped with aluminum foil which was secured with a rubber band. The capped tubes were loosely covered, autoclaved, and cooled. The caps were removed, and 60 ml of one-half strength Jensen mineral salts medium was asep-

TABLE 2. Legume hosts and control inoculant strains used to evaluate infectiveness of alfalfa nodule isolates

Host common name (genus and species)	Cultivar	Microsymbiont	
		Genus and species	Strain
Red clover (*Trifolium pratense* L.)	Florie	*Rhizobium leguminosarum* biovar *trifolii*	RT162P46[a]
Black medic (*Medicago lupulina* L.)[b]	Local ecotype	*R. meliloti*	102F34[a]
Birdsfoot trefoil (*Lotus corniculatus* L.)	Granger	*R. loti*	19BFT3[c]
Common bean [*Phaseolus vulgaris* (L.) Savi.][b]	Aurora	*R. leguminosarum* biovar *phaseoli*	127K12b[a]
Garden pea (*Pisum sativum* L.)	Little Marvel	*R. leguminosarum* biovar *viceae*	128C53[c]
Soybean (*Glycine max* Merr.)	Cutler	*Bradyrhizobium japonicum*	USDA 110[c]
Lupine (*Lupinus polyphyllus* L.)	Little Lulu	*Bradyrhizobium* sp. (*Lupinus*)	ATCC 10318[c]
Cowpea [*Vigna unguiculata* (L.) Walp.]	California blackeye no. 5	*Bradyrhizobium* sp. (*Vigna*)	32H1[c]

[a] Strains supplied by R. S. Smith (Nitragin Co.).
[b] Plant hosts nodulated by indigenous alfalfa isolates.
[c] Strains from the collection of H. J. Evans.

tically added to each tube. A single germinated seedling was placed in the vermiculite, the cap was replaced, and small flaps were cut in the cap with a sterile scalpel to allow emergence of the shoot. Each tube was covered with a sterile Whirl-Pak plastic bag (8 by 18 cm) (VWR Scientific, Inc., San Francisco, Calif.), which was securely fastened around the lower section of the tube. Tubes were then transferred to the growth chamber. At 5 days after planting, each seedling was inoculated with approximately 10^8 cells of a field isolate. Inoculated control strains listed in Table 2 were also applied as individual treatments to their respective host species. Noninoculated controls each received 1 ml of sterile YEM broth. Immediately after inoculation, the flapped hole surrounding the stem was packed with sterile cotton wool, and the plastic bag was again placed over the tube. At 7 days after planting, the ends of the plastic bags covering the large-seeded legume species (e.g., soybean, common bean) were cut off to allow shoot development, and the cut portion of the remaining bag was fastened closely around the stem. The experiment was terminated at 4 weeks after germination, and nodulation was assessed.

Subsequent tests were conducted to confirm the infectiveness of nodule isolates on the common bean. By using only the common bean as plant host, the first infectiveness experiment was repeated by using three additional replicates. Nodule reisolates were obtained from these plants for further experimentation.

An additional experiment was conducted to assess the possibility of contamination of the inoculant cultures and to determine if the native *Rhizobium* isolates were capable of reciprocal infectivity between common bean and alfalfa plants. A 2 × 6 factorial treatment combination was used with three replications. The treatments consisted of two host species (alfalfa and common bean) each receiving one of six inoculation treatments. The six inoculation treatments consisted of either the three parent cultures used for the initial infectivity test or the three reisolates of these strains from the common bean nodules produced in the experiment described previously. Control treatments consisted of effective commercial strains for the two plant species and noninoculated plants.

The purity of one of the parent cultures (191R) used in the previous infectiveness tests was examined further by testing the infectivity of 20 single-colony isolates from that culture on 20 bean and 20 alfalfa seedlings. Single colonies were obtained by serial dilution of a sample of the parent culture by using YEM broth containing 0.01% Tween 80 as the diluent. The suspensions were held on a Vortex mixer for 1 min between dilutions, and 0.1 ml of each dilution was streaked across a YEM agar plate. Broth cultures, produced from each of 20 single colonies, were used to inoculate one bean and one alfalfa plant. Seedlings were grown in a growth chamber by using the modified open-tube method and cultural conditions described previously.

RESULTS

During the initial purification of the field isolates, it was observed that many of the isolates produced small (1 mm) dry colonies which are typical of members of the genus *Bradyrhizobium* (21, 38). This colony type has also been observed with ineffective strains of various *Rhizobium* species (21). A few of the native isolates, however, produced large (2 to 4 mm) gummy colonies in 3 days, typical of fast-growing *Rhizobium* species.

The ineffective field isolates varied in their influence on media pH (Table 1). Unlike the acid reaction observed for the fast-growing commercial inoculant strains of *R. meliloti* (102F34) and *Rhizobium phaseoli* (127K12b), many of the field isolates had little effect on media pH. None of the field isolates grew on YEM agar at 39°C, but all of the commercial strains were able to produce colonies at this temperature. The mean generation times of the field *Rhizobium* isolates were longer and more variable (3.4 ± 0.1 to 9.9 ± 1.4 h) than those of the *R. meliloti* control strain 102F34 (1.5 ± 0.2 h).

To determine if the ineffectiveness of the field isolates was conditioned by the alfalfa cultivar, the relative effectiveness of 30 combinations of cultivars and field isolates (five cultivars and six strains) was evaluated. Seedling dry matter yield, the primary measure of effectiveness, was much lower for all plants inoculated with the *Rhizobium* field isolates in comparison with plants inoculated with the commercial strain 102F34 (Table 1). There were no differences between cultivars, and thus the shoot dry weight values were pooled for the various cultivars used. Nodules on plants receiving the most *Rhizobium* field isolates were typically small and white, however some strains (e.g., 166 and 191) produced larger, pink nodules often having a zone of brown deteriorating tissue at the base. This phenomenon has been described in detail previously for ineffective *M. sativa* nodules (36). A Gram stain revealed that these nodules also contained branched bacteroids which were similar to, yet slightly more compact than, those obtained from 102F34 nodules.

Since some of the native *Rhizobium* isolates exhibited

anomalous characteristics in pure culture (lack of either acid production, temperature tolerance, or fast growth on YEM growth media), it was of interest to verify that these strains were indeed *R. meliloti*. This was done by examining the infectivity of three of the field *Rhizobium* isolates on the various legume hosts listed in Table 2. All legume hosts were nodulated by their respective control strains, and none of the noninoculated controls was nodulated. Thus, it was concluded that growth conditions were appropriate for nodulation and that rhizobial contamination was not responsible for the results obtained. The only legumes which were nodulated by the *Rhizobium* field isolates were black medic (*M. lupulina*) and common bean [*Phaseolus vulgaris* (L.) Savi.]. All plants of these two species developed at least 15 nodules per plant. Nodule color observations of bean plants receiving *Rhizobium* field isolates revealed the same general response that was observed when alfalfa was nodulated with these isolates; strain 191R produced pink nodules, but the two other field isolates (082 and 172) produced smaller nodules with less pigmentation, i.e., either white or light brown. The bean nodules formed by native *Rhizobium* isolate 191R closely resembled the bean nodules formed by *Rhizobium leguminosarum* biovar *phaseoli* commercial strain 127K12b (spherically shaped, with white, ridged parenchyma tissue). One difference, however, was that the commercial strain produced nodules with a deeper red pigmentation. Nodules from both strains (127K12b and 191R) contained unbranched bacteroids. Because common bean is not typically nodulated by *R. meliloti* (37), the experiment was repeated by using common bean as the only plant host. Again, none of the noninoculated controls was nodulated, but all plants receiving the field isolates were nodulated. Preliminary experiments currently under way suggest that strain 191R is capable of fixing nitrogen on cultivars of common bean (data not shown).

To test for reciprocal infectivity of the *Rhizobium* field isolates and to investigate the possibility of contamination of the inoculant cultures with *R. phaseoli*, the infectiveness of the three native *Rhizobium* parent cultures and their respective reisolates from bean plant nodules was compared. Prolific nodulation was observed on both *M. sativa* and *P. vulgaris* regardless of the source of inoculant. No nodules were found on noninoculated controls. As was observed in the initial infectiveness experiment, nodule color and size were generally consistent for each strain. Thus, reciprocal infectivity of the native *Rhizobium* isolates between alfalfa and common bean was proven.

To test further the purity of one of the native isolate parent cultures (191), the infectiveness of 20 single-colony subcultures was evaluated on both alfalfa and common bean seedlings. Each subculture induced prolific nodulation on both plant species, with nodule color and morphology being consistent for each particular strain. Noninoculated controls did not nodulate.

DISCUSSION

The results of these experiments provided novel information on the cultural and symbiotic characteristics of several field isolates of *Rhizobium* found to be ineffective in N_2 fixation with *M. sativa*. Isolates of *R. meliloti*, like other fast-growing *Rhizobium* species, usually have a generation time of 2 to 4 h and are acid producers on YEM growth medium (38). In contrast, the slow-growing *Bradyrhizobium* species have a generation time greater than 6 h and usually produce an alkali reaction when grown on conventional media. Interestingly, the ineffective nodule isolates from *M. sativa* examined in this study exhibited characteristics of both groups by showing atypical combinations of growth rate and acid production characteristics when grown on conventional media. The common practice of categorizing *Rhizobium* species based on their growth rate and reaction in conventional media has been useful and generally consistent within certain cross-inoculation groups (18, 28, 38); however, exceptions are becoming increasingly common (10, 19, 22, 24, 34). These exceptions are important, since they decrease the utility of these methods of classification and expand the diversity of *Rhizobium* species.

The relatively slow growth rate of some of the field isolates used in these experiments is an interesting phenomenon. The cause for slower growth in some species of *Rhizobium* is obscure; however, it may be related to carbohydrate metabolism (19). Since the native isolates in this study infected only hosts of fast-growing *Rhizobium* species, it was initially thought that the slow growth rate of these organisms may have been due to some peculiar inadequacy of the YEM growth medium. To evaluate this possibility, experiments were conducted measuring growth on various carbon sources and diverse complex media (B. D. Eardly, Ph.D. thesis, Oregon State University, Corvallis, 1985). No evidence of stimulated growth was observed.

It has been well documented that ineffectiveness or partial effectiveness in the *M. sativa-R. meliloti* symbiosis may be conditioned by the host cultivar used (9, 14, 15, 17, 23). Results of this study suggest that the alfalfa cultivars used had no influence on the symbiotic ineffectiveness expressed by the nodule isolates from *M. sativa*.

The apparent promiscuity of the native *Rhizobium* isolates in terms of reciprocal infectiveness between common bean (*P. vulgaris*) and alfalfa (*M. sativa*) is of particular interest. *M. sativa* is rarely nodulated by *Rhizobium* species from outside its own cross-inoculation group (18, 37), with few exceptions reported (35, 40). In the study by Wilson (40), it was observed that an isolate from *M. sativa* formed nodules on *Phaseolus coccineus* L. (scarlet runner bean). The observation in the current study, that all three of the selected *M. sativa* native isolates shared an infective relationship between *M. sativa* and *P. vulgaris*, is important because it obscures the established host specificity associated with the *R. meliloti* cross-inoculation group. Substantiation of this observation by using several diverse *P. vulgaris* genotypes may reveal host specificity within that species.

Indirect evidence supporting the unusual reciprocal infectivity observed can be found. For example, Graham and Parker (18) observed that, out of 12 host species examined, *P. vulgaris* appeared to be the most promiscuous host in that it was nodulated by members of six of the seven groups of root nodule bacteria examined. *R. meliloti* was the only species which did not nodulate *P. vulgaris*. Because *P. vulgaris* is such a receptive host, it would seem a likely candidate to participate in a cross-infective relationship with a member of the highly specific *R. meliloti* cross-inoculation group.

Cross-infectiveness between other legume species has previously been attributed to the transfer of genetic material between microsymbionts (20). Dunican and Cannon (11) proposed that, under certain soil conditions, the transfer of effectivity via plasmids may occur, however there are few data supporting this hypothesis. A nucleic acid hybridization study by Gibbins and Gregory (16) indicated a degree of similarity between *R. meliloti* and *R. phaseoli*. Although interspecies transfer of genetic material in soils has not been

documented, this would provide one explanation for the observed promiscuity.

Because of the symbiotic promiscuity of the native isolates in this study, it is unclear whether the isolates are more closely related to *R. meliloti* or to *R. phaseoli*. Clearly, additional studies using more definitive methods of identification (21) are required to resolve this question.

Because of the high degree of host specificity which is typically expressed by *R. meliloti*, ineffective nodulation in alfalfa has traditionally been attributed either to incompatible strains of *Rhizobium* from within the *R. meliloti* cross-inoculation group (7) or to the influence of acid soil conditions, which are thought to adversely affect the retention of symbiotic effectiveness traits (9). Since *Rhizobium* species from outside the *R. meliloti* cross-inoculation group rarely nodulate *Medicago* species (18, 38, 40), it is reasonable to assume that little concern was ever given to the possibility that ineffective nodulation of field-grown alfalfa may be due to nodulation by a promiscuous *Rhizobium* species from outside the *R. meliloti* group. The results of this study, however, suggest that promiscuous *Rhizobium* species which can ineffectively nodulate *M. sativa* may exist. If substantiated elsewhere, these results could provide an alternative explanation for the reports of ineffective nodulation of alfalfa from various locations around the world (9).

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