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Bacterial Growth Rates and Competition Affect Nodulation and Root Colonization by *Rhizobium meliloti*

DE-MING LI AND MARTIN ALEXANDER*

Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York 14853

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The addition of streptomycin to nonsterile soil suppressed the numbers of bacterial cells in the rhizosphere of alfalfa (*Medicago sativa* L.) for several days, resulted in the enhanced growth of a streptomycin-resistant strain of *Rhizobium meliloti*, and increased the numbers of nodules on the alfalfa roots. A bacterial mixture inoculated into sterile soil inhibited the colonization of alfalfa roots by *R. meliloti*, caused a diminution in the number of nodules, and reduced plant growth. *Enterobacter aerogenes*, *Pseudomonas marginalis*, *Acinetobacter* sp., and *Klebsiella pneumoniae* suppressed the colonization by *R. meliloti* of roots grown on agar and reduced nodulation by *R. meliloti*, the suppression of nodulation being statistically significant for the first three species. *Bradyrhizobium* sp. and "*Sarcina lutea*" did not suppress root colonization nor nodulation by *R. meliloti*. The doubling times in the rhizosphere for *E. aerogenes*, *P. marginalis*, *Acinetobacter* sp., and *K. pneumoniae* were less and the doubling times for *Bradyrhizobium* sp. and "*S. lutea*" were greater than the doubling time of *R. meliloti*. Under the same conditions, *Arthrobacter citreus* injured alfalfa roots. We suggest that competition by soil bacteria reduces nodulation by rhizobia in soil and that the extent of inhibition is related to the growth rates of the rhizosphere bacteria.

For members of the genus *Rhizobium* to induce nodulation on their leguminous hosts, the bacteria probably must grow in the rhizosphere as well as at the site on the root hair where invasion of the plant begins. In these environments, the rhizobia are probably growing at the expense of organic products excreted by the roots, but these bacteria must compete for the organic nutrients with other heterotrophic inhabitants of the rhizosphere.

In 1941, Nicol and Thornton (9) studied the role of competition among strains of *Rhizobium* spp. for nodule sites and the impact of such intraspecific competition on nodulation. Since that time, many investigations have dealt with intraspecific competition among strains used for inoculation or between inoculum strains and native rhizobia in soil (1, 3). In the rhizosphere, however, the dominant species are not members of the genus *Rhizobium*, and because the dominant bacteria in that environment are often able to grow rapidly (13), they are probably able to compete effectively with *Rhizobium* spp. and to keep the *Rhizobium* population small. The finding that *R. trifolii*, *R. phaseoli*, and *R. japonicum* (syn. *Bradyrhizobium japonicum*) multiplied in soil when other bacteria were suppressed but not when other genera were not inhibited suggests that competition for the supply of available organic nutrients is important, at least in soil apart from the roots (10). Nevertheless, such intergeneric competition has scarcely been explored. Hely et al. (6), however, suggested that the native microbial communities of certain Australian soils prevented the nodulation of *Trifolium subterraneum* because these microorganisms suppressed the colonization of the plant roots by *R. trifolii*. Several investigators have demonstrated that individual isolates of bacteria or fungi decrease the nodulation of aseptically grown subterranean and white clovers by *R. trifolii* (2, 5, 11) or the rate and extent of nodulation of aseptically grown alfalfa by *R. meliloti* (4). In two of these studies, it was shown that the organisms causing the suppression did not produce toxins against *R. trifolii* in culture (2, 11), indicating that the suppression of nodule formation resulted from competition with the root nodule bacteria; nevertheless, the inhibition could have resulted from a microbiologically induced change in the physiology of the host.

The present study was designed to determine whether competition between *Rhizobium* spp. and species from other bacterial genera occurs in soil and to establish the characteristics of bacteria that are effective competitors with rhizobia in the rhizosphere.

MATERIALS AND METHODS

Samples of Eel silt loam were collected to a depth of 15 cm. The soil was air dried and passed through a sieve (pore diameter, 2 mm). A K_2HPO_4 solution was thoroughly mixed with the sieved soil to increase the P concentration to 500 mg/kg of dry soil and to bring the pH to about 7.2. Soil samples (20 g each) were placed in tubes (diameter, 30 mm), and the soil was brought to a moisture content of 23% (wt/wt). When sterile soil was needed, 20-g portions in test tubes were adjusted to 23% (wt/wt) moisture and autoclaved for 1 h. The samples were incubated for 1 day at 30°C. The samples were then again autoclaved for 1 h. Portions of the autoclaved samples were plated on nutrient agar, and no colonies appeared after 5 days.

Seeds of alfalfa (*Medicago sativa* var. Iroquois) were surface sterilized by soaking them in a solution of 70% ethanol for 1 min, washing them with sterile distilled water, soaking them for 5 min in 50% sodium hypochlorite, and then washing them again in sterile water. The seeds were allowed to germinate in petri dishes containing water agar, and the seedlings were transferred to tubes (30 by 180 mm; three seedlings per tube) containing either soil or agar medium. The plants were grown in tubes of soil or agar. Each tube of agar contained 25 ml of modified Fahraeus medium (15). The agar medium was sterilized by autoclaving, inoculated when molten at 45°C with 10^5 bacterial cells per ml, and allowed to cool into slants, and then the seedlings were introduced into the tubes. The tubes containing soil or agar were placed randomly in a Biotronette Mark

* Corresponding author.

TABLE 1. Populations of *R. meliloti* and other bacteria in the rhizosphere of alfalfa grown in sterile soil after inoculation

Day after inoculation	No. of cells (10^6) per g of roots and adhering soil after inoculation with:			
	Bacterial mixture alone	*R. meliloti* alone	*R. meliloti* and bacterial mixture	
			Bacterial mixture[a]	*R. meliloti*[a]
7	117	116	90.5	0.30
14	148	156	138	2.80
21	170	167	145	1.00

[a] Numbers of the specified microorganisms in the rhizosphere.

III environmental chamber (Lab-Line Instruments, Inc., Melrose Park, Ill.) maintained at 27°C, and the plants were exposed to 14 h of light each day. Five replicate tubes were used for each time interval, each cell density, and each species in studies of population changes, and three replicates were used in investigations of nodule number. The dry weights of the plants were obtained by shaking the roots and washing them to remove soil particles, retrieving small roots that had been broken, drying the plants at 105°C for 24 h, and then weighing them.

To prepare inocula, *Arthrobacter citreus* 837, *Enterobacter aerogenes*, "*Sarcina lutea*," *Acinetobacter* sp., *Klebsiella pneumoniae*, and *Pseudomonas marginalis* were grown in nutrient broth. A strain of *R. meliloti* that was resistant to 1.0 mg of streptomycin (Sigma Chemical Co., St. Louis, Mo.) and 50 μg of erythromycin (Sigma) per ml and a strain of *Bradyrhizobium* sp. that nodulates cowpeas were grown in yeast extract-mannitol (YEM) broth (15).

After soil was inoculated, it was brought to 23% (wt/wt) moisture and mixed, and then seedlings were planted in the tubes. When the soil was amended with antibiotics, 10 mg of streptomycin sulfate was mixed with 10 g of nonsterile soil before the inoculum and water were added.

To count microorganisms in the rhizosphere, the plants were removed from the soil, shaken gently, and placed in100 ml of sterile distilled water in a bottle containing glass beads. The bottle was shaken 100 times by hand, dilutions were made, and cell numbers were counted by the pour plate method.

R. meliloti was distinguished from *Bradyrhizobium* sp., *Arthrobacter citreus*, and "*S. lutea*," because only *R. meliloti* formed colonies on YEM agar containing 1.0 mg of streptomycin and 50 μg of erythromycin per ml. *R. meliloti* colonies on agar were distinguished from those of *P. marginalis* because only the former produced pink colonies on YEM agar containing 0.5% congo red (Calbiochem-Behring, La Jolla, Calif.), whereas only the latter produced red colonies on nutrient agar containing 0.1% rose bengal (Calbiochem-Behring). In samples containing *R. meliloti* and *E. aerogenes*, *Acinetobacter* sp., or *K. pneumoniae*, counts were made on YEM agar plates incubated at 27°C and plates of nutrient agar incubated at 40°C because the latter three species but not *R. meliloti* grew at 40°C and all four grew at 27°C; the counts on nutrient agar gave the numbers of one of the species in the mixtures of two species, and the numbers of *R. meliloti* were obtained by subtracting the counts of the second species from the total counts. If the only bacterium present was *R. meliloti*, counts were made on YEM agar, and colonies were counted after 3 to 5 days of incubation at 30°C. In samples containing other bacteria, *R. meliloti* was enumerated on YEM agar supplemented with 1.0 mg of streptomycin and 50 μg of erythromycin per ml after incubation at 30°C for 5 to 7 days. Counts of indigenous soil bacteria were made on YEM agar, and the plates were incubated for 5 days at 30°C. The numbers of bacteria other than *R. meliloti* were then obtained by subtracting the counts on YEM agar containing the antibiotics. In samples without *R. meliloti*, the numbers of bacteria were counted on YEM agar after incubation for 1 to 2 days at 30°C.

To obtain a mixture of soil bacteria, dilutions of a soil suspension were plated on nutrient agar. After 3 days of incubation at 30°C, single colonies were picked. Eleven of these bacteria were grown in nutrient broth for 1 to 2 days and then mixed together to give final densities of each isolate of ca. 10^5 cells per ml. (This inoculum is hereinafter referred to as the bacterial mixture.) To show that the individual isolates did not produce toxins affecting *R. meliloti*, the bacteria were grown in nutrient broth for 2 days, the cultures were centrifuged at 7,700 × *g* for 10 min, and equal volumes of the supernatant fluid were mixed with distilled water to prepare YEM agar. Counts made after 3 days of *R. meliloti* grown in YEM broth were the same when YEM agar either with or without the bacterial excretions was used.

The doubling time of bacteria was measured during the logarithmic growth phase. For this purpose, the bacterial community on roots and adhering agar was counted by using nutrient agar. For measuring growth rates in culture, the bacteria were grown at 30°C in 200-ml Erlenmeyer flasks containing 100 ml of nutrient broth, except that the root nodule bacteria were grown in YEM broth, and changes in optical density were measured at 660 nm.

RESULTS

Sterile soil was inoculated with a mixture of soil bacteria, *R. meliloti* alone, or a mixture of soil bacteria and *R. meliloti*. The initial number of cells of each species used was 4×10^5 cells per g of dry soil. Alfalfa was grown in the soil. The data show that the bacterial mixture markedly suppressed the density of *R. meliloti* (Table 1). The counts of cells in the bacterial mixture were not significantly different statistically at 7, 14, or 21 days whether *R. meliloti* was added or not. On the other hand, the counts of *R. meliloti* were significantly less ($P < 0.05$) at 7, 14, and 21 days in the presence of the other bacteria than in their absence.

At 21 days, an average of 27 nodules was present per three plants if the soil received only *R. meliloti*, but an average of only 12 nodules was present per three plants if *R. meliloti* was inoculated along with the other bacteria. This difference was statistically significant ($P < 0.01$). Determinations of plant dry weights showed no significant differences among the three treatments at 7 and 14 days; however, the dry weights at 21 days were significantly less ($P < 0.05$) if the inoculum was the bacterial mixture without *R. meliloti* (8.70 mg per three plants) or was *R. meliloti* plus the bacterial mixture (7.77 mg) than if it was just *R. meliloti* (11.1 mg), although the differences between the first two treatments were not statistically significant. Thus, the bacterial mixture inhibited both nodulation and alfalfa growth.

Alfalfa was grown for 21 days in nonsterile soil either with 1 mg of streptomycin per g of dry soil or without streptomycin. The soil was inoculated with 1.1×10^6 *R. meliloti* cells per g of dry soil, and water was added to bring the moisture level to 23%. The seedlings were soaked in a suspension containing 1.1×10^6 cells of *R. meliloti* per ml and then planted in the soil. The changes in population in the several treatments are shown in Table 2. The value for total bacteria at 2 days in soil without streptomycin was high and not significantly different from the *R. meliloti* count in soil with

streptomycin because the count in one replicate was anomalously high and resulted in a large standard deviation. In the soil receiving streptomycin, the population of soil bacteria was significantly smaller than in the untreated soil at 2 and 4 days. However, the size of the bacterial population in the streptomycin-amended soil then increased, and the population sizes in the streptomycin-amended and unamended soils were statistically indistinguishable at 7, 14, and 21 days. The number of *R. meliloti* cells in soil treated with streptomycin was significantly greater than that in the unamended soil after all but one time period. It is interesting that the marked rise in the population of bacteria after 4 days in the presence of streptomycin was not correlated with a change in the size of the population of *R. meliloti*.

Measurements of the weights of plants grown in streptomycin-amended and unamended soils revealed no significant difference at 2, 4, 7, 14, and 21 days. These data suggest that the addition of streptomycin did not influence the growth of alfalfa, although it is not clear how long the streptomycin remained active. On the other hand, the plants grown in soil with and without streptomycin had 29 and 13 nodules, respectively, on 9 plants after 21 days, a difference that is statistically significant ($P < 0.01$).

Experiments were conducted to determine the effects of several bacteria individually on the multiplication of *R. meliloti* and on the nodulation of alfalfa grown on agar. Each tube with three plants was inoculated with the test bacterium only, *R. meliloti* 102, or both. In experiments in which plant weight was determined, uninoculated plants were also included. The inoculum was 5.0×10^6, 4.3×10^6, 7.5×10^6, 7.5×10^6, 15×10^6, 13×10^6, or 7.5×10^6 cells of *E. aerogenes*, *P. marginalis*, *Acinetobacter* sp., *K. pneumoniae*, *Arthrobacter citreus*, *Bradyrhizobium* sp., or "*S. lutea*," respectively, per tube. In these experiments, the *R. meliloti* inoculum was 20×10^6, 5.0×10^6, 7.5×10^6, 10×10^6, 13×10^6, or 75×10^6 cells per tube, respectively. Except for *Arthrobacter citreus*, the numbers of the test bacteria at each sampling date were the same in the absence or presence of *R. meliloti*; i.e., the latter had no statistically significant effects on six of the seven species of bacteria (Table 3). "*S. lutea*" was apparently partially suppressed at two of the sampling dates. Because of variability in counts among replicates of *Bradyrhizobium* sp., large differences in numbers were not statistically significant. However, appreciable and statistically significant effects were exerted by some of the test bacteria on *R. meliloti*. *E. aerogenes*, *P. marginalis*, *Acinetobacter* sp., *K. pneumoniae*, and *Arthrobacter citreus* significantly reduced the *R. meliloti* populations, except for the first sampling date for four of the five test bacteria and the second sampling date for *Acinetobacter* sp. However, *Bradyrhizobium* sp. and "*S. lutea*" had no detrimental effect on *R. meliloti* at any sampling date after 3 days. Moreover, the cell density of *R. meliloti* was higher in the presence of these two species than when the rhizobium cells were grown in association with any of the other five species.

Nodulation in the preceding experiment was determined on the 21-day-old plants, except that plants inoculated with *Bradyrhizobium* sp. were examined after 23 days. Because the experiments were conducted at different times and presumably under slightly different conditions, the numbers of nodules on plants inoculated with only *R. meliloti* varied in different experiments. Plants inoculated with test bacteria alone bore no nodules. Some of the test bacteria reduced nodulation, and others had no effect (Table 4). Thus, *E. aerogenes*, *P. marginalis*, and *Acinetobacter* sp. each reduced nodulation by *R. meliloti*, the effect being statistically significant. *K. pneumoniae* appeared to have a smaller

TABLE 2. Populations of *R. meliloti* and total bacteria in the rhizosphere of alfalfa grown in nonsterile soil

Day after inoculation	No. of cells (10^6) per g of roots and adhering soil[a]			
	Soil with streptomycin		Soil without streptomycin	
	Total bacteria	*R. meliloti*	Total bacteria	*R. meliloti*
2	3.11 B	18.5 A	85.7 A	3.69 B
4	7.52 C	20.7 B	149 A	8.11 C
7	130 A	29.3 B	111 A	8.53 C
14	132 A	25.7 B	142 A	11.3 B
21	182 A	26.1 B	154 A	8.37 C

[a] Values in a row followed by different letters are significantly different ($P < 0.05$).

TABLE 3. Populations of *R. meliloti* and test bacteria in the rhizosphere of alfalfa grown on agar

Test bacterium and day after inoculation	No. of cells (10^6) per g of roots and adhering agar inoculated with[a]:			
	Test bacterium	*R. meliloti*	Mixed inoculum	
			Test bacterium	*R. meliloti*
E. aerogenes				
3	33.5 A	0.70 B	33.4 A	0.44 B
7	44.7 A	32.8 A	41.7 A	3.51 B
14	62.0 A	73.4 A	58.2 A	5.81 B
21	54.7 A	31.5 B	52.4 AB	6.00 C
P. marginalis				
3	21.7 A	2.91 B	19.9 A	0.26 C
7	23.6 A	21.1 A	22.7 A	8.71 B
14	36.7 A	39.7 A	34.5 A	10.1 B
21	40.7 B	52.3 A	40.9 AB	13.6 C
Acinetobacter sp.				
3	18.9 A	0.20 B	19.2 A	0.14 B
7	28.3 A	0.51 B	23.1 A	0.52 B
14	56.7 A	47.9 A	50.8 A	2.90 B
21	97.9 A	92.8 A	76.0 A	7.81 B
K. pneumoniae				
3	3.60 A	0.80 B	4.61 A	0.30 B
7	11.5 A	9.70 A	13.7 A	3.30 B
14	23.7 A	21.7 A	13.8 A	8.71 B
21	48.4 A	24.5 A	24.5 A	6.33 B
Arthrobacter citreus				
3	0.81 A	0.50 A	0.60 A	0.21 A
7	6.52 A	1.11 B	2.71 B	0.64 C
14	16.3 A	6.04 B	6.71 B	1.43 C
21	23.5 B	33.1 A	18.6 B	8.53 C
Bradyrhizobium sp.				
3	0.49 C	0.72 B	0.48 C	0.91 A
10	1.81 A	27.0 A	12.2 A	41.0 A
15	30.1 A	67.7 A	14.5 A	58.7 A
23	47.6 A	81.0 A	17.6 A	66.1 A
"*S. lutea*"				
3	1.70 A	1.24 A	2.14 A	1.32 A
7	7.80 A	11.8 A	8.93 A	12.0 A
14	19.2 A	15.1 A	14.1 A	16.3 A
21	10.1 A	29.4 A	9.61 A	31.1 A

[a] Values in a row followed by different letters are significantly different ($P < 0.05$).

TABLE 4. Number of nodules on alfalfa inoculated with *R. meliloti* and several test bacteria and growth rates of bacteria in axenic culture on surfaces of alfalfa roots grown on agar

Test species	Nodule no. on nine plants inoculated with:		Ratio of B/A	Generation time (h)
	R. meliloti only (A)	*R. meliloti* and test bacterium (B)		
E. aerogenes	29	15[a]	0.52	4.05
P. marginalis	152	92[a]	0.61	4.13
Acinetobacter sp.	47	30[b]	0.64	4.40
K. pneumoniae	52	41[c]	0.79	6.34
Bradyrhizobium sp.	36	38[c]	1.06	13.6
"*S. lutea*"	52	56[c]	1.08	10.6
R. meliloti	NA[d]	NA	NA	8.38

[a] Significantly different ($P < 0.01$) from number on plants inoculated with *R. meliloti* alone.
[b] Significantly different ($P < 0.05$).
[c] Not significantly different.
[d] NA, Not applicable.

effect, although it was not statistically significant. No influence was exerted by *Bradyrhizobium* sp. and "*S. lutea*."

The doubling times of *E. aerogenes*, *P. marginalis*, *Acinetobacter* sp., *K. pneumoniae*, *Arthrobacter citreus*, "*S. lutea*," *Bradyrhizobium* sp., and *R. meliloti* in nutrient broth were 4.42, 4.47, 5.24, 6.19, 7.30, 12.6, 14.0, and 7.72 h, respectively. Growth rates were also determined in the rhizosphere of alfalfa grown on agar. At the times that the bacteria were growing logarithmically, the seedlings were 3 to 10 days old. A logarithmic rate of increase was noted when the cell densities were about 10^6 to 10^7 cells per g of agar. The doubling times of the bacteria varied from 4.05 to 13.6 h (Table 4). *E. aerogenes*, *P. marginalis*, and *Acinetobacter* sp. grew most readily in the rhizosphere, and "*S. lutea*" and *Bradyrhizobium* sp. grew most slowly. *R. meliloti* grew at a rate between the two extremes.

The growth rates of individual bacteria were then compared with the effects of the bacteria on nodulation. For this purpose, the data are presented as nodulation ratios, the nodulation ratio being the ratio of the number of nodules on plants inoculated with both *R. meliloti* and the test bacteria to the number of nodules on alfalfa inoculated with only *R. meliloti*. The two species that grew more slowly than *R. meliloti* ("*S. lutea*" and *Bradyrhizobium* sp.) had little or no effect on nodulation (Table 4). In contrast, four of the five species of bacteria that grew more quickly than *R. meliloti* suppressed nodulation. The sole anomaly was *Arthrobacter citreus*, which had a nodulation ratio of 0.10 and a doubling time of 8.36 h (data not shown). Tests of *Arthrobacter citreus* revealed that culture filtrates made after its axenic growth had no effect on *R. trifolii*. However, because more than half of the roots had turned brown 8 days after transplanting the seedlings to agar inoculated with *Arthrobacter citreus*, the anomalous nodulation ratio may have resulted from root injury. Ignoring the value for *Arthrobacter citreus*, the suppression of nodulation was directly related to growth rates of the bacteria that had shorter generation times than *R. trifolii*.

DISCUSSION

Bacteria other than rhizobia and bradyrhizobia increase in numbers more markedly and attain greater densities in the rhizosphere of legumes than do the root nodule bacteria (7, 8). This suggests that the former are good competitors with the latter and prevent the rhizobia from obtaining a large part of the excreted carbon to support replication. Abundant evidence exists for competition among strains of *Rhizobium* spp. (9, 12, 14), but these bacteria grow slowly and do not account for the large increase in the size of the bacterial community as roots emerge from the seed and extend through the soil.

An effect of bacteria other than *Rhizobium* spp. on the nodulation of legumes has been demonstrated by a number of investigators. Thus, Anderson (2) reported in 1957 that the addition of several species of bacteria onto the roots of white clover grown on agar reduced the number of nodules and sometimes prevented nodulation by *R. trifolii*. More recent studies confirmed these observations, the later investigations involving suppression by *Azospirillum* spp. of nodulation of white and subterranean clovers by *R. trifolii* (11) and inhibition by *Erwinia herbicola* of the nodulation of alfalfa by *R. meliloti* (4). In several instances, the bacteria suppressing nodulation on these plants did not produce toxins active against the rhizobia (2, 11), so the effect was one associated either with competition or a physiological change induced in the plant. Fungi may also decrease nodulation (2, 5), but they may not be as important as bacteria because of their lower frequency in the rhizosphere.

The present study shows that growth rates greatly affected the outcome of competition between *R. meliloti* and other bacteria. Bacteria with slow growth rates did not significantly affect *R. meliloti*, whereas those that multiplied rapidly were good competitors. A relationship was also noted between the growth rate of bacteria and the number of nodules formed on alfalfa by *R. meliloti*. Bacteria that grew faster than *R. meliloti* reduced the number of nodules, and those that grew more slowly had no such effect.

The results from the tests with sterile and nonsterile soil further support the view of the importance of competition. Thus, a mixture of soil bacteria significantly reduced the density of *R. trifolii* when added to sterile soil. Similarly, suppressing bacteria in nonsterile soil for at least 4 days by the addition of streptomycin allowed a streptomycin-resistant strain of *R. meliloti* to reach higher densities than it would have attained in the absence of the antibiotic, and the amendment also led to an increase in the number of nodules. Although the antibacterial effect of streptomycin had dissipated after 4 days, possibly because of destruction of the antibiotic or the growth of resistant bacteria, nodulation was still promoted.

If competition is important under field conditions, it may be alleviated to some extent by using rapidly growing strains of *Rhizobium* spp. as inoculants. Competition may also be alleviated by use of a mixed inoculum containing bacteria that produce antibacterial antibiotics and *Rhizobium* spp. that are resistant to the antibiotics or by additions of inhibitors to which the inoculum strain, but no other rhizosphere bacteria, is resistant.

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