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An Experimental Test of the Rhizopine Concept in *Rhizobium meliloti*

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In some *Rhizobium*-legume symbioses, compounds known as rhizopines are synthesized by bacteroids and subsequently catabolized by free-living cells of the producing strain. It has been suggested than rhizopines act as proprietary growth substrates and enhance the competitive ability of the producing strain in its interactions with the diverse microbial community found within the rhizosphere. Wild-type, rhizopine-producing *Rhizobium meliloti* L5-30 and mutant L5-30 strains deficient for either rhizopine synthesis or catabolism were inoculated onto lucerne host plants in competition experiments. These experiments demonstrated that no apparent advantage resulted from the ability to synthesize a rhizopine, whereas the ability to catabolize rhizopine provided a clear advantage when an organism was in competition with a strain without this ability. The results suggest that when an organism is in competition with a catabolism-deficient mutant, the ability to catabolize rhizopine results in enhanced rates of nodulation. The results of the experiments were not consistent with the hypothesis that the sole role of rhizopines is to act as proprietary growth substrates for the free-living population of the producing strain.

In nitrogen-deficient environments the benefits accruing to the plant partner in rhizobium-legume associations are well understood. The plant gains from the bacterium's ability to convert atmospheric nitrogen into a form that can be assimilated. This additional nitrogen results in increased plant growth and reproductive success, which may in turn enhance the plant's competitive ability in its interactions with other members of the floral community. The benefits gained by the bacteria involved in these associations are much less well understood. This is particularly true in rhizobial species, such as *Rhizobium meliloti* and *Rhizobium leguminosarum*, in which the formation of a bacteroid represents an irreversible developmental event (7, 12). Thus, although the bacteroid stage is the stage responsible for nitrogen fixation, the formation of every non-reproductive bacteroid represents a loss to the bacterial population. The free-living bacteria inhabiting infection threads within nodules may obtain some degree of protection from biotic and abiotic environmental factors. However, a large fraction of the free-living rhizobial population is found in the rhizosphere (1). Competition in this nutrient-rich environment is intense as there is a diverse and abundant microbial community exploiting these resources.

One manner by which the rhizobial population could benefit from its association with plants would be if the bacteroid subpopulation produced a compound that could be exploited as a growth substrate exclusively by members of the free-living rhizobial population. The specific production of a growth substrate could confer a competitive advantage for the producers over other saprophytic microorganisms in the rhizosphere. Rhizopines may be such compounds.

Rhizopines (L-3-*O*-methyl-*scyllo*-inosamine, and *scyllo*-inosamine) are produced from plant precursors by bacteroids within nodules (3, 8, 10). Significantly, rhizopines can be catabolized only by the free-living cells of the producing strain (3). Not all strains of a species synthesize rhizopines (13). In an extensive survey, about 12% of the *R. meliloti* and *R. leguminosarum* strains tested were found to produce rhizopines (13). The genes for synthesis and catabolism of rhizopines are coincident and have been found only on the symbiotic plasmid. In *R. meliloti* the genes are closely linked, and rhizopine synthesis is regulated via the *nifA* gene (4). No species of bacteria other than some *Rhizobium* species has been found to have the ability to catabolize rhizopines (9). These factors suggest that rhizopines play a role in symbiosis, and it has been advocated that rhizopines represent proprietary growth substrates that enhance the population growth of the producing strain in the soil (5, 6).

The purpose of this study was to test the rhizopine concept in planta. From a naturally isolated L-3-*O*-methyl-*scyllo*-inosamine-synthesizing and -catabolizing *R. meliloti* strain, mutants were constructed by using a transposon-insertion technique. One of these mutants is defective for the ability to synthesize rhizopine (Mos^-), another mutant is unable to catabolize the rhizopine (Moc^-), and a third mutant contains the transposon but is unimpaired with respect to rhizopine synthesis or catabolism and served as a neutral control. Plants growing in pots were inoculated with each combination of wild type and mutant, and the relative frequencies of the strains were monitored through time.

MATERIALS AND METHODS

Strains and strain construction. The mutants produced for these experiments were all derived from *R. meliloti* L5-30 (Mos^+, Moc^+). The Mos^+ Moc^- mutant (strain 2129) and the Mos^+ Moc^+ neutral mutant (strain 2126) were prepared by mutating plasmid pPM1031 with Tn*5* and then incorporating the mutated fragment into the genome of *R. meliloti* L5-30 by marker exchange as previously described (3). The same procedure was used to obtain the Mos^- Moc^+ mutant (strain 2168), except that in this case the starting plasmid was pPM1062. The

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wild-type strain, L5-30, was modified so that it harbored plasmid pPH1JI, which was used in the other strains as part of the marker exchange protocol (strain 2338).

In vitro growth experiments. To determine if the Tn*5* insertion resulted in any significant change in the growth of the mutant strains, the exponential-phase growth rates of the three mutants and the wild-type strain were estimated for cultures growing in liquid medium and on agar medium. The medium used was TY (10 g of tryptone per liter, 5 g of yeast extract per liter, 0.9 g of $CaCl_2 \cdot H_2O$ per liter), and agar (15 g liter^{-1}) was added for the surface growth experiments. Liquid culture experiments were carried out in 250-ml square bottles containing 50 ml of medium. For the surface cultures, 2 ml of liquid agar medium was spread onto sterile glass microscope slides and allowed to harden (11). The growth cultures were started from stationary-phase cultures, serially diluted to produce an initial density of about 10^4 cells ml^{-1}. The inoculum (100 μl) was spread uniformly on the agar-coated slides. All cultures were incubated at 28°C, and the liquid cultures were shaken at 150 rpm. The cultures were sampled at regular intervals until the stationary phase was achieved (for liquid cultures, 56 h; for surface cultures, 72 h). Cell densities were determined by serial dilution and plating.

Single-strain nodulation experiments. Lucerne (variety Hunter River) seeds were germinated on agar. After germination (3 days) the seedlings were transplanted into steam-sterilized glass tubes (15 by 2 cm) containing a mixture of equal parts of sand and loam. The tubes were then randomly assigned to one of four groups. Immediately after transplanting, each seedling was inoculated with 100 μl (10^8 cells) of a stationary-phase culture of either the wild-type strain (2338) or the Moc$^-$ (2129), Mos$^-$ (2168), or neutral (2126) mutant. The inoculum was applied to the base of the stem. At regular intervals after inoculation 15 plants were randomly selected from each of the four groups of tubes and the number of nodules per plant was determined.

Plant culture. Experiments were carried out in tall-form 1-liter pots. The soil medium consisted of equal parts of loam and coarse sand supplemented with superphosphate (1.12 kg m^{-3}), $CaCO_3$ (600 g m^{-3}), and K_2SO_4 (600 g m^{-3}). The soil was steam pasteurized prior to use. The variety of lucerne (*Medicago sativa*) used was Hunter River, and Tamor was the variety of white clover (*Trifolium repens*) used. Seeds were sterilized by soaking them for 2 min in 100% ethanol and then for 2 min in a sodium hypochlorite solution (125 g liter^{-1}); this was followed by five rinses in sterile distilled water. Multiple seeds were sown per pot, and seedlings were thinned to one plant per pot prior to inoculation at 2 weeks after planting. The plants were watered as required with filtered water and were maintained under natural light conditions at 22 to 26°C during the day and 16 to 18°C at night.

Competition experiments. The basic design of the experiment consisted of inoculating plants with equal numbers of one of the mutant strains and the wild-type strain. The frequencies of both cell types were then monitored through time by isolating bacteria from nodules taken from the lucerne host plant. The following three strain combinations were tested: Moc$^-$ mutant and wild-type strain (strain 2129 versus strain 2338), Mos$^-$ mutant and wild type strain (strain 2168 versus strain 2338), and neutral mutant and wild-type strain (strain 2126 versus strain 2338). For each of the three strain combinations the changes in the relative frequencies of the mutant and wild-type strains were monitored in three ways.

The first method, designated the lucerne harvest experiment, involved inoculating a large number of pots containing young lucerne plants with one of the three strain combinations. At regular intervals, a number of pots were randomly selected, the plants were removed, and the nodules were harvested. This harvest method allowed the relative frequencies of the mutant and wild-type strains in the nodule-inhabiting population of bacteria to be estimated.

The second method, the lucerne resown experiment, involved inoculating another group of pots containing the lucerne host with one of the three strain combinations. At regular intervals, a number of pots were randomly selected, and the plants, including the roots, were removed and discarded. The soil from each pot was mixed and returned to the original pot, and the pots were then resown with lucerne seed. After 5 weeks had elapsed, the host plants were removed and the nodules were harvested. In the second planting, the lucerne served as a "trap" host. The bacteria present in the soil were responsible for nodule formation on these plants.

For the third method, referred to as the clover-lucerne experiment, a group of pots containing white clover were inoculated with one of the three strain combinations. At regular intervals, a number of pots were randomly selected and the clover plants were removed and discarded. The soil was returned to the pots, and the pots were resown with lucerne seed. The lucerne plants were allowed to grow for 5 weeks, and then they were removed and their nodules were harvested. The purpose of the clover was to deny the bacteria a host while allowing for a nonspecific rhizosphere effect. As described above, the lucerne served as a trap host.

Initiation of the experiment. A total of 450 pots were seeded with lucerne, and 180 pots were seeded with white clover. At 2 weeks after seeding equal numbers of plants were inoculated with one of the three strain combinations. The mutant and wild-type strains were cultured separately in TY for 48 h. Each of the mutant strain cultures was thoroughly mixed with an equal volume of the wild-type culture. For each of the three strain combinations, the relative frequencies of the mutant and wild-type strains in the initial inoculum mixture were determined by replicated serial dilution and plating ($n = 6$). Each plant was inoculated with 1 ml (10^9 cells) of the appropriate strain combination. The inoculum was applied to the base of the stem.

TABLE 1. Sample sizes in the in planta competition experiments and the proportions of nodules cooccupied by mutant and wild-type strains

Expt	Mutant compared with wild type	Mean no. of plants per sample	Mean no. of nodules sampled per plant	Mean % of nodules with both strains
Lucerne harvest	Neutral	10	19	9.6
	Mos$^-$	7	21	10.2
	Moc$^-$	9	22	9.3
Lucerne resown	Neutral	8	6	2.2
	Mos$^-$	8	11	2.1
	Moc$^-$	8	9	2.4
Clover-lucerne	Neutral	7	7	3.7
	Mos$^-$	7	9	4.0
	Moc$^-$	7	11	1.5

Nodule and bacterial sampling. Nodules were removed from the plants such that a small amount of root remained on either side of the nodule. The nodules were surface sterilized for 2 min in 2% sodium hypochlorite, and this was followed by five washes in sterile distilled water. The nodules were placed individually in the wells of a microtiter plate containing 40 μl of sterile distilled water per well. They were then crushed with a sterile wooden applicator stick, and 30 μl of the mixture from each well was streaked onto a TY plate containing streptomycin (250 μg ml^{-1}). The plates were incubated for 3 to 4 days at 28°C. From each plate 10 well-isolated colonies were transferred with a toothpick onto a TY plate containing streptomycin, and a TY plate containing streptomycin and kanamycin (250 μg ml^{-1}). These plates were incubated at 28°C for 3 to 4 days, and the numbers of Kan$^+$ (mutant) and Kan$^-$ (wild-type) colonies were recorded. The purposes of the streptomycin were to select for the L5-30 strains and to prevent the growth of potential *R. meliloti* contaminant strains. Less than 2% of naturally isolated strains of this species are streptomycin resistant ($n = 117$) (unpublished data).

Statistical analysis. A plant was considered the experimental unit. For each plant the total number of wild-type and mutant colonies was determined and expressed as the ratio of wild-type colonies to total colonies. The total number of L5-30 colonies per plant equaled the number of colonies tested per nodule (10) times the number of nodules examined per plant. For various reasons it was not always possible to examine the same number of pots per sample or to test the same number of nodules per plant. The average number of plants examined per sample and the mean number of nodules examined per plant are presented in Table 1. Overall, less than 10% of the nodules examined were occupied by more than one strain (Table 1).

The analyses used were one-way analysis of variance and least-squares linear regression. For every treatment combination the data were first analyzed to determine if there was any significant change through time in the frequency of the wild-type strain following inoculation. If no such trend was detected, then the average frequency of the wild-type strain after inoculation was compared with the frequency of the wild-type strain in the inoculum.

Test for frequency dependence. Competitive interactions are often frequency dependent (that is, a situation in which the fitness advantage conferred by a trait depends on the frequency of the trait in the population). To test for this possibility, an additional experiment was conducted. TY cultures of the wild-type strain and the Moc$^-$ strain were mixed to produce three inocula that varied in the proportion of wild-type cells present; these inocula contained 5, 36, and 85% wild-type cells. These cultures were used to inoculate pots containing five lucerne seedlings (10^9 cells per pot). At 4, 7, and 13 weeks after inoculation three pots were selected at random from each treatment, the nodules were harvested from the plants, and the relative frequencies of the wild-type strain were determined in the manner described above. The data were analyzed by a two-way analysis of variance.

RESULTS

In vitro growth experiments. In liquid culture the Moc$^-$ mutant (2129) was the only mutant to grow significantly more slowly than the wild-type strain (2338) and exhibited a growth rate 7% less than the growth rate of the wild-type strain (Table 2) ($P < 0.001$). This result was not observed for the Moc$^-$ mutant growing on a surface culture. On surfaces, no significant differences in the growth rates of the four strains were detected (Table 2) ($P > 0.5$).

TABLE 2. Exponential-phase growth rates of the mutant and wild-type L5-30 strains in TY under different culture conditions

Strain	Phenotype	Growth rate (h^{-1})	SE (h^{-1})	% of wild-type value
Liquid cultures				
2338	Wild type	0.33	0.003	100
2126	Neutral	0.33	0.004	100
2168	Mos^-	0.33	0.002	101
2129	Moc^-	0.30	0.002	93
Surface cultures				
2338	Wild type	0.28	0.014	100
2126	Neutral	0.27	0.014	97
2168	Mos^-	0.30	0.011	107
2129	Moc^-	0.29	0.012	102

Single-strain nodulation experiments. The number of nodules per plant increased through time in a similar manner for all four strains ($P < 0.001$) (Fig. 1). No differences between the strains in the number of nodules recovered per plant were detected ($P > 0.65$), nor was any interaction between strain and time detected ($P > 0.32$).

In planta competition experiments. When the wild-type strain was in competition with the neutral mutant, a significant decline in the level of the wild-type strain over time was observed in the nodule-inhabiting population of bacteria (Fig. 2A) ($P < 0.001$). In the lucerne resown experiment the frequency of the wild-type strain in the nodules of the trap hosts also declined when the wild-type strain was in competition with the neutral mutant (Fig. 2B) ($P < 0.022$). The rates of decline were comparable in the two experiments (0.7 and 0.8%/week, respectively). In the clover-lucerne experiment, no change in the frequency of the wild-type strain in the nodules of the trap hosts was detected when the wild-type strain was in competition with the neutral mutant (Fig. 2C) ($P > 0.54$).

The frequency of the wild-type strain also declined when this strain was in competition with the Mos^- mutant. This decline was observed in the lucerne harvest experiment (Fig. 3A) ($P <$ 0.001) and in the nodules of the trap host in the lucerne resown experiment (Fig. 3B) ($P < 0.006$). The frequency of the wild-type strain declined at comparable rates in the two experiments (0.6 and 0.7%/week, respectively). However, in the clover-lucerne experiment no change in the frequency of the wild-type strain in the nodules of the trap hosts was detected (Fig. 3C) ($P > 0.08$).

Quite different results were obtained in the experiments involving the wild-type strain and the Moc^- mutant. Subsequent to inoculation no change in the frequency of the wild-type strain in the nodule population of bacteria was detected through time ($P > 0.66$). However, the average frequency of

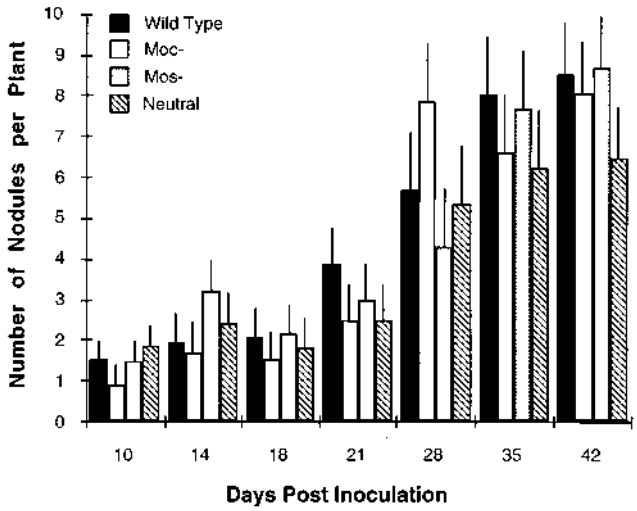

FIG. 1. Accumulation on lucerne plants of nodules produced by wild-type *R. meliloti* L5-30 and different rhizopine Tn*5*-induced mutants. The number of nodules per plant increased in a linear manner (note that the *x* axis is not an interval scale). The vertical lines indicate the 95% confidence limits of the means.

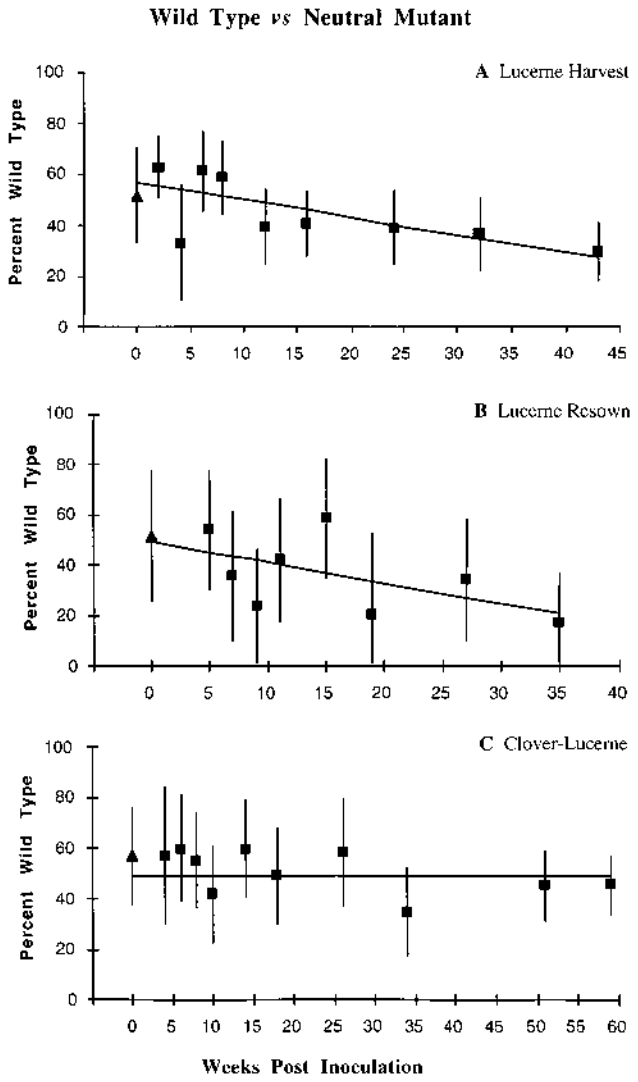

FIG. 2. Change in frequency through time of rhizopine-producing wild-type *R. meliloti* (Mos^+ Moc^+) cells occupying nodules when they were in competition with the neutral Tn*5*-induced mutant (Mos^+ Moc^+). (A) Frequency of the wild-type strain in the nodule population of the primary lucerne crop. (B) Frequency of the wild-type strain in the nodules of the lucerne trap hosts when the primary crop was lucerne. (C) Frequency of the wild-type strain in the nodules of lucerne trap hosts when the primary crop was clover. See Materials and Methods for details of the three experimental treatments. The solid triangle indicates the frequency of the wild-type strain in the inoculum. The solid squares indicate the frequency of the wild-type strain in free-living cells occupying nodules of lucerne hosts. The vertical lines indicate the 95% confidence limits of the mean frequency. The solid lines indicate the change through time in the frequency of the wild-type strain after inoculation.

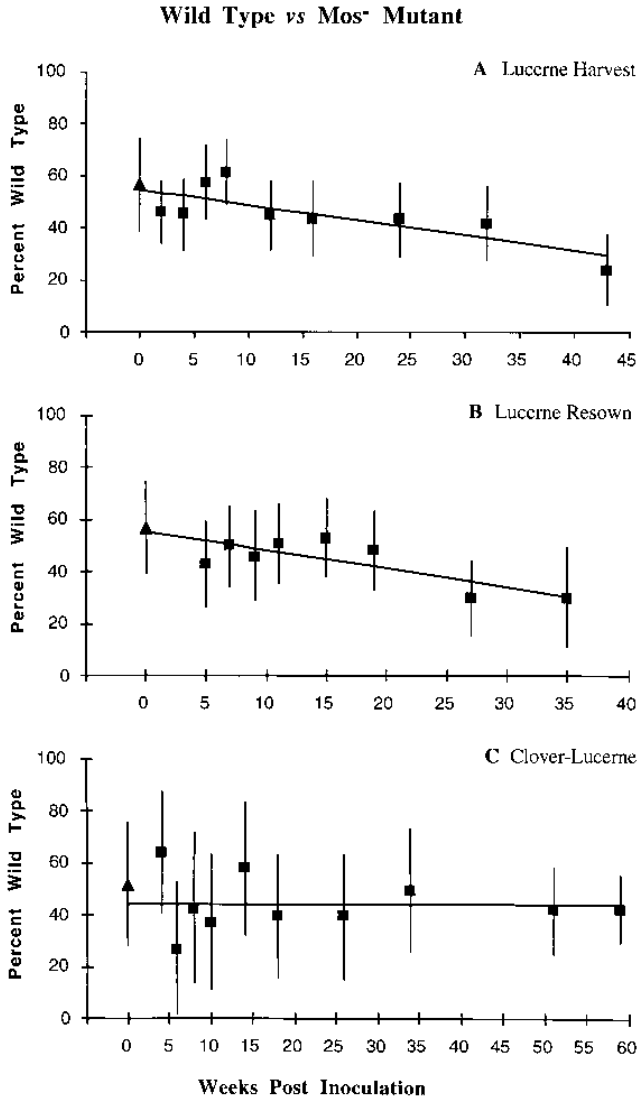

FIG. 3. Change in frequency through time of rhizopine-producing wild-type *R. meliloti* (Mos^+ Moc^+) cells occupying nodules when they were in competition with the Mos^- Tn*5*-induced mutant (Mos^- Moc^+). (A) Frequency of the wild-type strain in the nodule population of the primary lucerne crop. (B) Frequency of the wild-type strain in the nodules of the lucerne trap hosts when the primary crop was lucerne. (C) Frequency of the wild-type strain in the nodules of lucerne trap hosts when the primary crop was clover. See Materials and Methods for details of the three experimental treatments. The solid triangle indicates the frequency of the wild-type strain in the inoculum. The solid squares indicate the frequency of the wild-type strain in free-living cells occupying nodules of lucerne hosts. The vertical lines indicate the 95% confidence limits of the mean frequency. The solid lines indicate the change through time in the frequency of the wild-type strain after inoculation.

the wild-type strain after inoculation was 73%, a value significantly greater than the frequency in the inoculum (47%) (Fig. 4A) ($P < 0.001$). Comparable changes in the frequency of the wild-type strain when it was in competition with the Moc^- mutant were seen in the nodules of the trap hosts in the lucerne resown experiment (Fig. 4B). After inoculation, no change in the frequency of the wild-type mutant over time was detected ($P > 0.54$). However, the average frequency of the wild-type strain across all samples subsequent to inoculation (67%) was significantly greater than the frequency of this strain in the inoculum (47%) ($P < 0.015$). Again, similar changes in the frequency of the wild-type strain when it was in competition with the Moc^- mutant occurred in the clover-lucerne experiment (Fig. 4C). Following inoculation no change in the frequency of the wild-type strain in the nodules of the trap hosts was observed ($P > 0.40$). The average frequency of the wild-type strain after inoculation was 72%, a value significantly greater than the frequency of this strain in the inoculum (47%) ($P < 0.002$).

Test for frequency dependence. On average, the wild-type strain was recovered at a significantly higher frequency with respect to the Moc^- mutant than would be expected on the

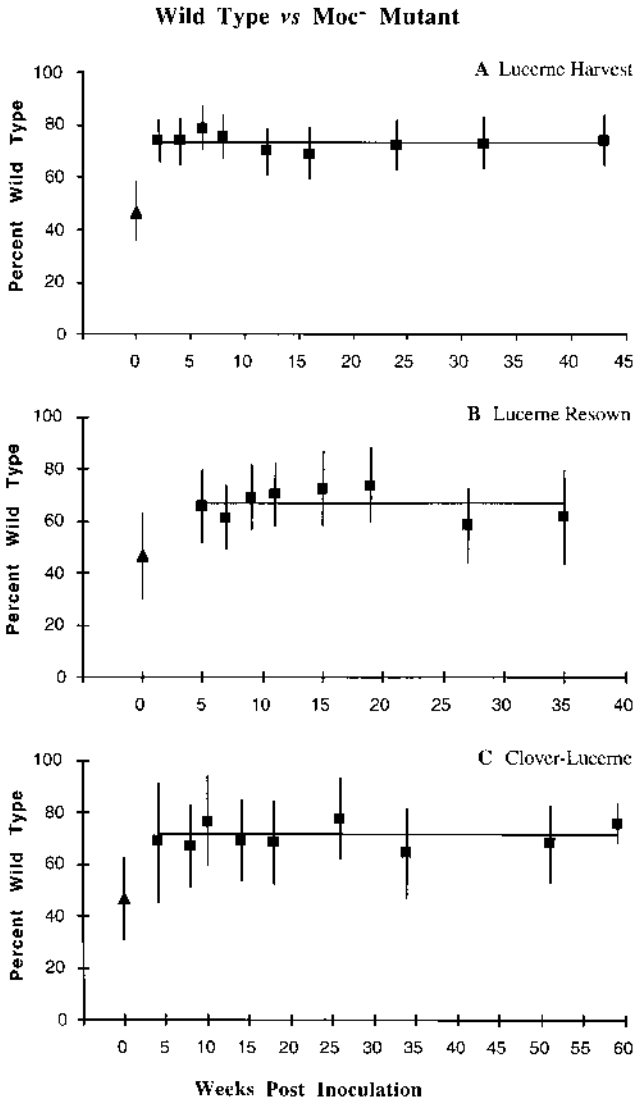

FIG. 4. Change in frequency through time of rhizopine-producing wild-type *R. meliloti* (Mos^+ Moc^+) cells occupying nodules when they were in competition with the Moc^- Tn*5*-induced mutant (Mos^+ Moc^-). (A) Frequency of the wild-type strain in the nodule population of the primary lucerne crop. (B) Frequency of the wild-type strain in the nodules of the lucerne trap hosts when the primary crop was lucerne. (C) Frequency of the wild-type strain in the nodules of lucerne trap hosts when the primary crop was clover. See Materials and Methods for details of the three experimental treatments. The solid triangle indicates the frequency of the wild-type strain in the inoculum. The solid squares indicate the frequency of the wild-type strain in free-living cells occupying nodules of lucerne hosts. The vertical lines indicate the 95% confidence limits of the mean frequency. The solid lines indicate the change through time in the frequency of the wild-type strain after inoculation.

basis of its frequency in the inoculum ($P < 0.001$). The frequencies of the wild-type strain in the inocula were expected to be 5, 36, and 85%, but the observed frequencies of the wild-type strain were 48, 77, and 99.5%, respectively. The frequency of the wild-type strain did not change with the time of harvest ($P > 0.45$), and no initial frequency–time of harvest interaction was detected ($P > 0.39$).

In all experiments nodules occupied by both wild-type and mutant strains represented less than 10% of the nodules examined (Table 1). Thus, the results presented in Fig. 2 through 4 not only represent the relative frequencies of wild-type cells but also reflect the relative frequencies of wild-type nodules (that is, nodules in which the wild-type strain represents at least 90% of the population of free-living cells inhabiting the nodule) (10 colonies were examined per nodule).

DISCUSSION

Expected experimental outcomes. What results would be expected from the competition experiments if the function of rhizopine is to provide a proprietary growth substrate for the free-living population of the producing strain? The following scenarios assume that the Tn*5* insertion has no nonspecific effect on the fitness of the mutants (that is, that mutant and wild-type cells have identical demographic characteristics except with respect to rhizopine synthesis and catabolism). Given this assumption, the frequencies of wild-type and mutant cells recovered from the nodules of the trap hosts (in lucerne resown and clover-lucerne experiments) should reflect the frequencies of wild-type and mutant cells in the soil. Thus, the trap hosts are assumed to provide a sample of the soil population at a particular point in time. In reality, this point in time was about 3 weeks. The time from sowing to harvest was 5 weeks, and it took somewhat less than 1 week for germination to occur, while any nodules formed within about 1 week of harvest were too small to be sampled.

In wild-type strain versus neutral mutant experiments the frequency of the wild-type strain should equal its frequency in the inoculum and should not change through time as the two strains are identical with respect to rhizopine synthesis and catabolism. Similar results are expected in wild-type strain versus Mos$^-$ mutant experiments. While the Mos$^-$ mutant is incapable of synthesizing rhizopine, it is capable of catabolizing the rhizopine produced by the wild-type strain. The total amount of rhizopine being synthesized by the bacteroid population should be reduced because a certain fraction of bacteroids are incapable of synthesis. However, the reduction in the amount of rhizopine present in the rhizosphere should affect the wild-type and Mos$^-$ strains equally. Thus, while the total population of free-living cells might be smaller, the relative frequencies of the two strains should equal their frequencies in the inoculum and should not change through time. These results are expected in lucerne harvest, lucerne resown, and clover-lucerne experiments for both wild-type strain versus neutral mutant and wild-type strain versus Mos$^-$ mutant competition experiments.

In experiments involving the wild-type and Moc$^-$ strains, the initial frequency of the wild-type strain in the soil and nodule populations should equal its frequency in the inoculum. Once symbiotically effective nodules are formed (1 to 2 weeks), the bacteroids of both strains should start producing rhizopine, but only the wild-type strain should be able to exploit the rhizopine as an additional carbon source. As a result, the frequency of the free-living wild-type cells should increase through time in the rhizosphere, and this should in turn be reflected by an increase in the frequency of the wild-type strain in the nodule-inhabiting population of bacteria.

In clover-lucerne experiments no bacteroids are formed and no rhizopine is produced while the clover is present. As a result, no change in the frequency of the wild-type strain relative to the Moc$^-$ mutant should occur no matter how long the strains are in the presence of the nonhost species. Each crop of lucerne trap hosts is in the soil for less than 5 weeks. In addition to the time required for germination, there is the time required for symbiotically effective nodules to develop and for rhizopine synthesis to commence. Furthermore, the nodules formed on the trap hosts should be small and few in number given the time available. As a consequence, little rhizopine should be produced, and at most only a small increase in the frequency of the wild-type strain relative to its frequency in the inoculum is expected. This effect, if detectable, should be the same for each crop of trap hosts, and therefore no change in the frequency of the wild-type strain relative to the Moc$^-$ mutant over time is expected.

Some differences between the results of lucerne harvest and lucerne resown experiments might be expected when the wild-type strain is competing with the Moc$^-$ mutant. In both experiments rhizopine is produced in the presence of the primary lucerne crop, and therefore the frequency of the wild-type strain in the rhizosphere is expected to increase through time. However, in lucerne harvest experiments the rate at which the wild-type strain increases in frequency in the nodule population may be influenced by the rate of nodule formation on the primary crop. Nodules do not accumulate on a host in an unrestricted manner; the rate of nodule formation declines as the number of nodules per plant increases (2). As a consequence, the frequency of the wild-type strain in the nodule population of bacteria at a particular point in time may not reflect the frequency of the wild-type strain in the rhizosphere at that time. The extent to which this influences the results depends on the rates at which old nodules senesce and new nodules are formed. This phenomenon should not be a factor in lucerne resown experiments. The second crop of lucerne does not acquire a sufficient number of nodules in the brief time that it is in the soil.

Observed experimental outcomes. The results suggest that the Tn*5* insertion did not affect the demographic characteristics of the mutant strains (except rhizopine synthesis and catabolism). The results of the in vitro growth experiments suggest that the Tn*5* insertion had no effect on the exponential-phase growth rates of the mutant strains when they were growing on surfaces. The environmental conditions of the surface experiments are most like those of a soil environment.

The results of the single-strain nodulation experiments suggest that the Tn*5* insertion did not affect the nodulation ability of any of the mutant strains relative to the wild-type strain. All strains formed nodules on the host plant at the same rate, and over 5 weeks there was no indication of any decline in the rate of nodule formation (note that the x axis in Fig. 1 is not an interval scale). The results of these experiments suggest that an important underlying assumption of the experiments is justified; this assumption is that the relative frequencies of wild-type and mutant cells recovered from the nodules of the trap host reflect the relative frequencies of mutant and wild-type cells in the soil. Furthermore, these results suggest that density dependence in the rate of nodule formation is unlikely to be a significant factor in determining the number of nodules recovered from plants used as trap hosts as, on average, there were less than 10 nodules per trap host (Table 1).

Contrary to expectations, the wild-type strain declined in frequency with respect to both the neutral and Mos$^-$ mutants

in the lucerne harvest and lucerne resown experiments. The reasons for the decline in the frequency of the wild-type strain are unknown. The decline in the frequency of the wild-type strain was not observed in the clover-lucerne experiment, a result which suggests that when no host plant is present, the survival and/or growth characteristics (fitness) of the wild-type strain and the neutral or Mos$^-$ mutants in the soil are similar. The different outcomes of these experiments imply that the decline in the frequency of the wild-type strain was due to events occurring in the nodule-inhabiting populations of free-living cells rather than in the soil population. Although the decline in the frequency of the wild-type strain is unexplained, the results demonstrate that small changes through time (0.7%/week) in the frequency of the wild-type strain relative to the mutants could be detected in these experiments. The ability to observe small changes in frequency is relevant to the outcome of the wild-type strain versus Moc$^-$ mutant experiments.

In all in planta experiments the average frequency of the wild-type strain in nodules relative to the Moc$^-$ mutant ($\sim$70%) was significantly greater than would be expected on the basis of the frequency of the wild-type strain in the inoculum (47%). The increase in frequency was observed within 2 weeks after inoculation, and no further change in the frequency of the wild-type strain through time was detected. These results are contradictory to the predicted outcome of these experiments. If the rhizopine acts solely as a growth substrate, then the frequency of the wild-type strain should gradually and continuously increase through time. An increase in the frequency of the wild-type strain should not be observed within 2 weeks after inoculation. At this time symbiotically effective nodules are only just appearing, and these nodules are small and few in number. Thus, very little rhizopine is being produced. Furthermore, no increase in the frequency of the wild-type strain through time was observed.

The results of the wild-type strain versus Moc$^-$ mutant experiment designed to test for frequency dependence support the results of the main experiment. The wild-type strain was consistently recovered at a higher frequency from the nodules than would be expected on the basis of its frequency in the inoculum (Fig. 4). This result was observed within 4 weeks after inoculation, and the frequency of the wild-type strain did not change thereafter.

These experiments demonstrated that there is a clear fitness advantage associated with the ability to catabolize a rhizopine. However, none of the results are consistent with an advantage resulting from the rhizopine acting as a specific growth substrate. The results do suggest that when a strain with the ability to catabolize rhizopine is in competition with a strain lacking this ability, the strain with the ability nodulates plants at a higher rate. The enhanced nodulation ability of the Moc$^+$ (wild-type) strain is not a by-product of the presence of Tn*5* in the mutants. The results of the single-strain nodulation experiments do not support such a conclusion. The fitness advantage occurs only when the strain is in competition with the Moc$^-$ mutant. A competitive advantage resulting from enhanced nodulation ability, rather than a change in numbers in the soil, would produce outcomes consistent with those observed in these experiments. In particular, these outcomes are consistent with the observation that the advantage is expressed within 2 weeks of host plant inoculation.

The results of our experiments demonstrate that it is unlikely that the only role of rhizopines is to act as proprietary growth substrates. Rather, the ability to catabolize a rhizopine appears to enhance the rate at which a strain is able to form nodules when it is in competition with a strain that is unable to catabolize a rhizopine. The mechanism(s) responsible for this enhanced nodulation ability is unknown and will require investigation.

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REFERENCES

1. **Beringer, J. E., N. Brewin, A. W. B. Johnston, H. M. Schulman, and D. A. Hopwood.** 1979. The *Rhizobium* legume symbiosis. Proc. R. Soc. Lond. B Biol. Sci. **204:**219–233.
2. **Caetano-Anollés, G., and P. M. Gresshoff.** 1991. Plant genetic control of nodulation. Annu. Rev. Microbiol. **45:**345–382.
3. **Murphy, P. J., N. Heycke, Z. Banfalvi, M. E. Tate, F. J. de Bruijn, A. Kondorosi, J. Tempé, and J. Schell.** 1987. Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the Sym plasmid. Proc. Natl. Acad. Sci. USA **84:**493–497.
4. **Murphy, P. J., N. Heycke, S. P. Trenz, P. Tatet, F. J. de Bruijn, and J. Schell.** 1988. Synthesis of an opine-like compound, a rhizopine, in alfalfa nodules is symbiotically regulated. Proc. Natl. Acad. Sci. USA **85:**9133–9137.
5. **Murphy, P. J., and C. P. Saint.** 1992. Rhizopines in the legume-*Rhizobium* symbiosis, p. 377–390. *In* D. P. S. Verma (ed.), Molecular signals in plant-microbe communication. CRC Press, Boca Raton, Fla.
6. **Murphy, P. J., M. Wexler, W. Grezemski, J. P. Rao, and D. M. Gordon.** 1995. Rhizopines—their role in symbiosis and competition. Soil Biol. Biochem. **27:**525–529.
7. **Paau, A. S., C. B. Bloch, and W. J. Brill.** 1980. Developmental fate of *Rhizobium meliloti* bacteroids in alfalfa nodules. J. Bacteriol. **143:**1480–1490.
8. **Rao, J. P., W. Grezemski, and J. P. Murphy.** 1995. *Rhizobium meliloti* lacking *mos*A synthesizes the rhizopine *scyllo*-inosamine in place of 3-*O*-methyl-*scyllo*-inosamine. Microbiology **141:**1683–1690.
9. **Rossbach, S., G. Rasul, M. Schneider, B. Eardly, and F. J. de Bruijn.** 1995. Structural and functional conservation of the rhizopine catabolim (*moc*) locus is limited to selected *Rhizobium meliloti* strains and unrelated to their geographical origin. Mol. Plant Microb. Interact. **8:**549–559.
10. **Saint, C. P., M. Wexler, M. Tate, and J. P. Murphy.** 1993. Characterization of genes for synthesis and catabolism of a new rhizopine induced in nodules by *Rhizobium meliloti* Rm220-3: extension of the rhizopine concept. J. Bacteriol. **175:**5205–5215.
11. **Simonsen, L.** 1991. Dynamics of plasmid transfer on surfaces. J. Gen. Microbiol. **136:**1001–1007.
12. **van den Bos, R. C., and W. J. Broughton.** 1981. Viability of *Rhizobium leguminosarum* endosymbionts in different stages of development. Arch. Microbiol. **129:**349–353.
13. **Wexler, M., D. M. Gordon, and J. P. Murphy.** 1995. The distribution of inositol rhizopine genes in *Rhizobium* populations. Soil Biol. Biochem. **27:** 531–537.