Construction and Environmental Release of a *Sinorhizobium meliloti* Strain Genetically Modified To Be More Competitive for Alfalfa Nodulation

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Highly efficient nitrogen-fixing strains selected in the laboratory often fail to increase legume production in agricultural soils containing indigenous rhizobial populations because they cannot compete against these populations for nodule formation. We have previously demonstrated, with a *Sinorhizobium meliloti* PutA[−] mutant strain, that proline dehydrogenase activity is required for colonization and therefore for the nodulation efficiency and competitiveness of *S. meliloti* on alfalfa roots (J. I. Jiménez-Zurdo, P. van Dillewijn, M. J. Soto, M. R. de Felipe, J. Olivares, and N. Toro, Mol. Plant-Microbe Interact. 8:492–498, 1995). In this work, we investigated whether the *putA* gene could be used as a means of increasing the competitiveness of *S. meliloti* strains. We produced a construct in which a constitutive promoter was placed 190 nucleotides upstream from the start codon of the *putA* gene. This resulted in an increase in the basal expression of this gene, with this increase being even greater in the presence of the substrate proline. We found that the presence of multicopy plasmids containing this *putA* gene construct increased the competitiveness of *S. meliloti* in microcosm experiments in nonsterile soil planted with alfalfa plants subjected to drought stress only during the first month. We investigated whether this construct also increased the competitiveness of *S. meliloti* strains under agricultural conditions by using it as the inoculum in a contained field experiment at León, Spain. We found that the frequency of nodule occupancy was higher with inoculum containing the modified *putA* gene for samples that were analyzed after 34 days but not for samples that were analyzed later.

Attempts to improve nitrogen fixation by introducing highly efficient *Rhizobium* strains often fail in soils that already contain indigenous rhizobial populations due to problems with competitiveness (6, 31, 32, 34). Despite being much less efficient at nitrogen fixation, the indigenous populations are generally better adapted and more persistent, with a higher level of infectivity, resulting in a higher level of occupancy of the nodules formed. Thus, to enhance nitrogen fixation, the strains used as inocula not only must fix nitrogen efficiently but also must be highly competitive. Genetic modifications can be used to increase the competitiveness of *Rhizobium* strains. An example of the successful use of this strategy is the improvement of *Rhizobium etli* competitiveness, even under agricultural conditions, achieved by introducing genes encoding trifoliotoxin (23, 24).

Our laboratory has studied the genetics of competitiveness in *Sinorhizobium meliloti*. The *putA* gene, which encodes proline dehydrogenase, the enzyme catalyzing the oxidation of proline to glutamate, is one of the genetic loci thought to be involved in this symbiotic property. The ability of a PutA[−] mutant to colonize the root surface was found to be impaired, as were nodulation efficiency and competitiveness (11, 12). The root exudates of the host plant of *S. meliloti*, alfalfa (*Medicago*

sativa L.), contain proline and compounds such as betaines and stachydrine that release proline upon degradation (20). Mutants impaired in stachydrine utilization have also recently been shown to be affected in their ability to colonize alfalfa roots (20). These observations suggest that proline may be an important energy source for the bacteria during the first stages of the infection process. However, mutants with mutations in either the *putA* gene or the stachydrine utilization (*stcD*) gene continue to produce effective nodules (12, 20). Therefore, proline does not appear to be an important energy source for nitrogen fixation within bacteroids.

Complementation of the PutA[−] mutant with a cosmid containing the wild-type *putA* gene did more than merely restore the competitiveness of the mutant to wild-type levels; it actually rendered the complemented mutant more competitive than the wild type (11). This suggested that manipulation of the expression of this gene could be used to improve the competitiveness of *S. meliloti* strains in field conditions. In *S. meliloti*, the *putA* gene is transcriptionally activated by proline and the resulting PutA protein serves as an autogenous repressor (30). In this work, we constructed a plasmid containing a *putA* gene that had high basal expression levels but was also inducible by proline. We evaluated the competitiveness of *S. meliloti* strains harboring this plasmid in microcosm and contained field release experiments. The increased competitiveness of *S. meliloti* containing the modified *putA* construct is discussed.

MATERIALS AND METHODS

Bacterial strains, media, growth conditions, plasmids, and DNA manipulation. The bacterial strains and plasmids used in this study are listed in Table 1.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> DH5 α	Host for cloning	Bethesda Research Laboratories
<i>S. meliloti</i>		
GR4	Wild type: Nod ⁺ Fix ⁺	5
GRM8	Derivative of GR4 cured of cryptic plasmids	16
2011	Wild type: Nod ⁺ Fix ⁺	J. Denarié
M4	2011 with <i>gusA</i> gene integrated into a nonessential region between <i>recA</i> and <i>alaS</i> in the chromosome: Sm ^r	29
M401	M4 containing plasmid pBBRHG1; Sm ^r Hg ^r	This work
M403	M4 containing plasmid pBBRHG3; Sm ^r Hg ^r	This work
Plasmids		
pRK2013	Helper plasmid for mobilization: Km ^r	7
pUC18	Multicopy cloning vector; Ap ^r	37
pBluescript SK(+)	Multicopy cloning vector: Ap ^r	Stratagene
pBBR1MCS-2	Broad-host-range cloning vector: Km ^r	15
pJB3Km1	Broad-host-range IncP cloning vector: Ap ^r Km ^r	2
pGUS3	<i>pnfD-gusA</i> translational fusion in pB1101 (Clontech): Km ^r	9
pRG970	Broad-host-range <i>lacZ</i> fusion vector: Spc ^r Sm ^r	35
pPDH1	Derivative of pUC18 containing a 9-kb GRM8 chromosomal <i>EcoRI</i> fragment that contains the 3' end and downstream region of the <i>putA</i> gene Ap ^r	12
pPDH2	Derivative of pUC18 containing a 2.9-kb GRM8 chromosomal <i>EcoRI</i> fragment that contains the upstream region and 5' end of the <i>putA</i> gene: Ap ^r	12
pJZ301	Derivative of pRG970 containing a <i>putA-lacZ</i> transcriptional fusion: Spc ^r Sm ^r	12
pMH310	Derivative of pRG970 containing a <i>putA-lacZ</i> transcriptional fusion of the 190-bp region upstream from <i>putA</i> ; Spc ^r Sm ^r	30
pUT-Hg	Plasmid containing the mercury resistance cassette; Hg ^r	10
pJB3	<i>PstI</i> fragment from pJB3Km1 containing the kanamycin resistance gene inserted into pUC18: Km ^r Ap ^r	This work
pGP1	Derivative of pJB3 containing only the kanamycin resistance gene promoter (pKm) after autoligation of <i>XhoI</i> to <i>SalI</i> : Ap ^r	This work
pMJV1	1.5-kb fragment from pPDH2 containing the <i>putA</i> 3' end and 190-bp upstream region inserted in front of the pKm promoter of pGP1; Ap ^r	This work
pMJV2	1.7-kb fragment from pMJV1 containing pKm through to the <i>putA</i> 3' end inserted into pBBR1MCS-2: Km ^r	This work
pMH21	1.7-kb fragment from pMJV2 inserted into pBluescript SK(+); Ap ^r	This work
pMH210	1.7-kb fragment from pMH21 inserted into pRG970, resulting in a pKm- <i>putA</i> (upstream region)- <i>lacZ</i> transcriptional fusion; Spc ^r Sm ^r	This work
pMJV3	3-kb fragment from pPDH1 containing the <i>putA</i> 5' end inserted into pMJV2 resulting in a complete <i>putA</i> gene; Km ^r	This work
pBBRHG1	Derivative of pBBR1MCS-2 in which the kanamycin resistance gene was replaced by the mercury resistance cassette from pUT-Hg; Hg ^r	This work
pBBRHG3	Derivative of pMJV3 in which the kanamycin resistance gene was replaced by the mercury resistance cassette from pUT-Hg; Hg ^r	This work

Plasmid DNA was routinely isolated and manipulated by following standard protocols (26). *Escherichia coli* was grown at 37°C in Luria-Bertani medium (26). *S. meliloti* strains were grown at 28°C in tryptone-yeast medium (1) or in defined minimal medium (MM) (22). Antibiotics, fungicide, and mercury chloride were used as required at the following concentrations: ampicillin, 200 µg/ml; spectinomycin, 100 µg/ml; streptomycin, 50 µg/ml for *E. coli* and 250 µg/ml for *S. meliloti*; kanamycin, 50 µg/ml for *E. coli* and 180 µg/ml for *S. meliloti*; cycloheximide, 200 µg/ml; and mercury chloride, 3.75 µg/ml for *E. coli* and 1.5 µg/ml for *S. meliloti*.

***S. meliloti* strain used for genetic modification.** The M4 strain (Table 1) was chosen for genetic modification; this strain is tagged with the *gusA* gene in a nonessential region of the chromosome, between the *recA* and *alaS* genes, and is naturally resistant to streptomycin (29).

Plasmid stability. Stationary-phase cultures were diluted in fresh medium without selection to give an initial optical density of 0.03 at 620 nm and cultivated for 12 h (four generations). This process was repeated, and finally samples were serially diluted and plated onto solid media in the absence of selective drugs. One hundred colonies were chosen and picked onto plates with and without the selective antibiotics or mercury. The percentage of plasmid loss per generation was calculated with the formula $[1 - (Fr/Fi)/n] \times 100$, where *Fr* is the antibiotic resistance cell fraction after *n* generations and *Fi* is the initial fraction of resistant cells.

β-Galactosidase assays. β-Galactosidase activity was measured using the sodium dodecyl sulfate-chloroform method described by Miller (18), with *S. meliloti* cultures grown to exponential phase in MM broth with or without 0.2% (wt/vol) proline.

Microcosm studies. Ten alfalfa (*Medicago sativa* L. cv. Aragón) seedlings were grown in pots containing 125 g of soil, originating from Riego de la Vega (see "Field experiment plots and sites" below), mixed with 125 g of sterile sand. This soil is an eutric fluvisol (sand, 34%; silt, 44%; clay, 22%; pH 7.1; organic matter, 2.2%; total N, 0.18%; total organic carbon, 1.5%) with an indigenous *S. meliloti* population of 10² cells/g of dry soil. The pots were inoculated with 75 ml of water-inoculum mixture to obtain 10³ to 10⁴ CFU/g of soil mixture. Pots were covered with a layer of sterile perlite to avoid desiccation and placed in the growth chamber with a light-dark cycle consisting of 16 h of light and a temperature of 25°C followed by 8 h of darkness and a temperature of 18°C, with the humidity kept at 50%. Three replicates per treatment were performed. Roots were washed, and nodule occupancy was determined as described below.

Preparation of inoculum and seed coating for field release. To prepare the inoculum, cultures were grown to early stationary phase in tryptone-yeast medium supplemented with mercury chloride and appropriate antibiotics. The culture was washed and concentrated in MM to approximately 10¹¹ bacteria/ml. The inoculum was mixed with sterile peat at a 2:3 (vol/wt) ratio. Fifty grams of seeds (1 g = 440 seeds) was coated with 1 g of peat-inoculant mixture, 3 ml of sterile

FIG. 1. Map of the field site at Riego de la Vega, León, Spain. Three plots each were inoculated with M401 or M403 or were left untreated (NI).

water, and 1 ml of adhesive (40% [wt/vol] gum arabic solution), resulting in a density of approximately 10^5 bacteria/seed. The mixture was allowed to dry, and the seeds were then sown immediately, with 1.4 g of coated seeds per pot in each field plot.

Field experiment plots and sites. The field experiment was conducted at Riego de la Vega, León, Spain ($42^{\circ}24'N$, $5^{\circ}59'W$). This agricultural field has a long history of alfalfa, sugarbeet, and barley crops. The indigenous *S. meliloti* population in this field site before the release experiment was 10^2 cells/g of dry soil (as determined using the most-probable-number method described by Brockwell [4]).

The field site was partitioned into nine plots, each covering an area of 2.25 m², separated from each other by 1.5 m (Fig. 1). We partially buried nine plant pots in each plot, filling the pots with the displaced soil. Three plots were treated with M401 (an M4 derivative containing plasmid pBBRHG1 [Table 1]) as a control, three were treated with M403 (an M4 derivative containing plasmid pBBRHG3, which carries the *putA* gene under the control of the pKm promoter [Table 1]), and the remaining three were left untreated as controls. The plants were watered according to weather conditions, as they would be in a typical agricultural field, and were not specifically subjected to drought conditions. On 25 May 1999, the pots in these plots were sown either with seeds coated with sterile peat (untreated) or with seeds coated with M401 or M403. Measures were taken to protect the alfalfa from birds and foraging animals. Weeds were removed regularly by hand. For sampling, one pot from each plot (three pots per treatment) were removed for further analysis. Soil samples were obtained as described under "Persistence" below. To determine nodule occupancy, root nodules were separated from roots prior to root maceration. The isolated nodules were surface sterilized for 5 min in 0.15% (wt/vol) mercury chloride solution, washed in sterile water, and stained as described under "Nodule occupancy" below.

Persistence. The persistence of the inoculum was determined on three different surfaces: bulk soil (soil not associated with alfalfa roots), rhizosphere soil (soil adhering to the root surface), and rhizoplane (the root surface). Samples from each surface type were serially diluted in sterile water and plated on MM supplemented with 20 μ g of X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) (Apollo Scientific Ltd., Stockport, United Kingdom) per ml, streptomycin, mercury chloride, and cycloheximide. Persistence was assessed by counting the blue *S. meliloti* colonies that developed. To obtain rhizosphere soil, alfalfa roots were washed in sterile phosphate-buffered saline (PBS) (140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM NaH₂PO₄) for 20 min. The buffer used for washing was then centrifuged for 5 min at $8,700 \times g$ at 4°C. The pellet corresponded to rhizosphere soil, 1 g of which was used to produce serial 1:10 dilutions, which were then plated. To determine the persistence of bacteria on the rhizoplane, roots from which rhizosphere soil had been removed were washed three more times with 50 ml of sterile PBS for 10 min each. The nodules were removed from these roots, and nodule occupancy was assessed. We then ground 0.5 g (wet weight) of root in 4.5 ml of sterile mortar. The resulting suspension was serially diluted (1:10) in sterile water and plated.

Nodule occupancy. We incubated the nodules, overnight at 37°C in the dark, in 1 mM X-Gluc in 50 mM sodium phosphate buffer (pH 7.5)–1% (wt/vol) sodium dodecyl sulfate. Nodule occupancy by modified and wild-type organisms was assessed by counting blue (*gusA*-containing strains) and white nodules, respectively. Nodules from each of the three replicates per treatment were pooled prior to β -glucuronidase activity assays. The total number of nodules analyzed at each time is indicated in Tables 3 and 5. The 95% confidence

TABLE 2. Expression of *putA::lacZ* transcriptional fusions in *S. meliloti* GRM8

Strain	β -Galactosidase activity (U) ^a	
	Without proline	With proline
GRM8/pRG970	7 $\pm$ 0.6	9 $\pm$ 2
GRM8/pJZ301	40 $\pm$ 5	194 $\pm$ 20
GRM8/pMH210	254 $\pm$ 20	1,117 $\pm$ 20
GRM8/pMH310	8 $\pm$ 1	8.5 $\pm$ 1.5

^a Mean values and standard errors were calculated from at least three independent experiments. All data except those for GRM8/pMH210 have been previously reported (30).

intervals were estimated by multiplying standard errors of the proportions by 1.96. For statistical analyses, z scores for proportions were determined and contrasted to confidence levels of 0.05.

RESULTS AND DISCUSSION

Modification of *putA* gene expression. Previous studies (11) have suggested that the competitiveness of *S. meliloti* strains could be increased by increasing expression of the *putA* gene. To test this hypothesis, we inserted a promoter that would ensure high basal *putA* gene expression. As we have previously shown (30), the promoter sequences required for expression of the *putA* gene are located between 190 and 290 nucleotides upstream from the translation start site. The 190-nucleotide upstream region present in pMH310 was insufficient to drive *putA* gene expression (Table 2). The constitutive promoter of the kanamycin resistance gene (*nptII*) was placed 190 nucleotides upstream from the translation start site of the *putA* gene, and a transcriptional fusion between this combination and *lacZ* was produced in pRG970 to give pMH210 (Table 1; Fig. 2). β -Galactosidase activity in the presence and absence of proline was measured for *S. meliloti* strain GRM8, which harbored pMH210 (Table 2). These results were compared with those previously obtained in the laboratory (30). In the absence of proline, pMH210 gave six-times-higher levels of expression than the wild-type promoter region present in pJZ301. In the

FIG. 2. DNA fragments fused to the reporter gene *lacZ* of pRG970. Open boxes and arrows indicate DNA sequences of *putA*, the filled box indicates the promoter of the kanamycin resistance gene, and the filled circles indicate the proline regulation region. pJZ301 carries a *putA::lacZ* transcriptional fusion containing the wild-type promoter region (12). pMH210 carries a *putA::lacZ* transcriptional fusion containing the constitutive promoter of the kanamycin resistance gene 190 nucleotides upstream from the *putA* gene. pMH310 carries a *putA::lacZ* transcriptional fusion containing only the 190-nucleotide region upstream from the *putA* gene (30).

TABLE 3. Nodule occupancy in microcosm experiments

Time (no.) after inoculation	Inoculum	Drought stress		Control	
		% Blue nodules ^a	Total no. of nodules	% Blue nodules	Total no. of nodules
1	M4	67.2 ± 12.0 a	58	49.4 ± 7.7 b	164
	M4/pBBR1MCS-2	57 ± 11.0 ab	79	62 ± 8.8 a	116
	M4/pMJV3	90 ± 7.8 cd	63	47.6 ± 8.8 b	124
2	M4	92.5 ± 5.0 d	100	98.6 ± 1.5 e	213
	M4/pBBR1MCS-2	93.6 ± 5.0 d	98	82.7 ± 5.3 c	197
	M4/pMJV3	97 ± 4.4 de	58	91.9 ± 4.6 d	136

^a Three replicates per treatment were prepared, and nodules were pooled before the analysis. Nodules occupied by M4 or its derivatives stain blue with X-Gluc (for both single and double occupancy). Standard errors shown are calculated at a confidence level of 95%. Means followed by different letters are significantly different, with a *P* value of ≤0.05.

presence of proline, the level of expression of *putA* by pMH210 was four times higher than that in the absence of this amino acid.

Microcosm experiments. Proline exuded by alfalfa plants may be an important energy and carbon source for *S. meliloti* during the colonization and infection process (11, 12, 20). Thus, an enhanced ability to metabolize this energy source may constitute a metabolic advantage, rendering the strain more competitive. The properties of the promoter region from pMH210 coupled with a functional *putA* gene may confer such a metabolic advantage on the bacteria. We therefore fused the promoter region of pMH210 to the remainder of the *putA* gene, to obtain the plasmid pMJV3 (Table 1). Soybean and alfalfa plants have been shown to accumulate proline in their nodules when grown under salt or drought stress (8, 13, 14). This may result in an increase in the availability of proline within root exudates. If this is indeed the case, then any metabolic advantage conferred by the higher level of *putA* gene expression from pMJV3 should be observed more clearly under drought conditions. We therefore performed microcosm studies under these conditions with an inoculum containing pMJV3.

We grew alfalfa seedlings in nonsterile soil originating from an agricultural field in the province of León in the north of Spain. The soil was inoculated with *S. meliloti* strain M4 harboring either pMJV3 or the parental *putA*-less vector, pBBR1MCS-2, in quantities 10 to 100 times the size of the local *S. meliloti* population. To simulate drought conditions, the plants were watered every 2 days with a 22% volume of water per g of soil mixture (drought stress), or, as a control, the plants were watered daily with 30% (vol/wt) water. Regardless of the inoculum used, plants subjected to simulated drought conditions had approximately half the dry shoot weight of well-watered plants (36) and showed half the number of the nodules elicited under control conditions.

Under simulated drought conditions, 90% of the nodules analyzed in the first month after inoculation were occupied by the strain containing pMJV3, which carries a complete *putA* gene under control of the pKm promoter (Table 3). This proportion was significantly higher than that obtained with either of the control strains under the same conditions. However, the percentage of nodules occupied by the control strains increased over time, whereas the percentage occupied by the M4/pMJV3 strain remained constant. As a result, under simulated drought conditions, by the second month, the control

strains occupied a proportion of the nodules similar to that obtained with the strain containing pMJV3.

In contrast, in well-watered plants, M4/pMJV3 occupied only 47.6% of the nodules in the first month. This proportion was similar to that obtained under the same conditions with the M4 control strain. After 2 months in well-watered pots, the proportion of nodules occupied by each inoculum increased. However, under these conditions, the presence of pMJV3 does not seem to affect competitiveness.

Overall, the results of these microcosm studies indicated that pMJV3 allowed M4 to occupy a higher proportion of nodules only under simulated drought conditions and only during the first month. Plasmid pMJV3 provides strain M4 with a quicker but transient advantage in nodule occupancy. Nevertheless, by shortening the period of time required to occupy most of the nodules elicited on alfalfa roots, the inoculant strain could manifest its symbiotic traits within the plant earlier.

Construction of the inoculum for field release. According to regulatory requirements, the kanamycin gene of pMJV3 was replaced with a mercury resistance cassette, giving pBBRHG3. As a control, the kanamycin gene of pBBR1MCS-2 was also replaced with the same cassette to give pBBRHG1 (Table 1). These two plasmids, pBBRHG1 and pBBRHG3, were introduced into *S. meliloti* strain M4 by conjugation, resulting in strains M401 (control strain) and M403 (carrying in the plasmid vector a *putA* gene under the control of the pKm promoter), respectively.

Environmental release. (i) Inoculation. The positive results obtained with the modified *putA* gene in the microcosm experiments conducted under simulated drought conditions led us to study the effect of this gene in a contained field release experiment. M401 and M403 were applied during the spring of 1999 to an agricultural field in Riego de la Vega, León, Spain. The soil of this field site was previously used for the microcosm experiments.

In our study, both the amount of inoculum applied and the size of the indigenous *S. meliloti* population in the soils used were similar to those in other field experiments involving genetically modified *S. meliloti* strains. These experiments typically involved the use of 10⁵ to 10⁶ bacteria per seed or per g of soil to inoculate soils containing indigenous *S. meliloti* populations of up to 10³ bacteria per g of soil (3, 17, 19, 21, 27, 28).

(ii) Persistence. The inocula used in our field experiment persisted throughout the experiment in rhizosphere soil and on

FIG. 3. Persistence of the control strain M401 (○) and the PutA-overproducing strain M403 (■) in bulk soil (A), in rhizosphere soil (B), and on the rhizoplane (C). The values shown for day 0 correspond to the CFU of each strain released per seed. Mean values and error values were calculated from three independent measurements. Error bars indicate standard errors, at a confidence level of 95%.

the rhizoplane, with more than 10^2 bacteria per g (wet weight) of soil or root (Fig. 3). However, M403 was less persistent than M401 on these surfaces. This is probably because pBBRHG1 was more stable in M401 than pBBRHG3 was in M403 (the proportions of plasmid-containing cells after eight generations

TABLE 4. Nodule occupancy in the field experiment

Time (days)	M401		M403	
	% Blue nodules ^a	Total no. of nodules	% Blue nodules	Total no. of nodules
34	71.0 ± 7.1 a	155	87.3 ± 5.6 b	134
98	82.9 ± 4.8 b	240	80.6 ± 4.8 b	258
159	79.9 ± 6.0 ab	174	88.8 ± 4.0 b	233

^a Nodules occupied by M401 or M403 (single or double occupancy) stain blue. Standard errors (95% confidence level) are indicated. Means followed by different letters are significantly different, with a *P* value of ≤0.05.

were 88.9 and 76.4%, respectively; the corresponding values for M4/pBB1MCS-2 and M4/pMJV3 were 100 and 95.8%, respectively). However, neither type of inoculum persisted well in bulk soil (Fig. 3A). Persistence assays performed with bulk soil surrounding the pots sown with seeds coated with M401 or M403 also showed that neither of these types of inoculum was present (data not shown). The higher persistence within the pots of the inoculum in rhizosphere soil and on the rhizoplane is not surprising, as the bacteria coating the seeds probably multiply preferentially along the growing root surface.

(iii) Nodule occupancy. The frequencies of nodule occupancy by M401 and M403 in the field experiment are indicated in Table 4. These results are very similar to those obtained with M4/pBB1MCS-2 and M4/pMJV3 in the microcosm experiments performed under simulated drought conditions. In the field, M403, which contains the modified *putA* gene, occupied a larger proportion of the nodules than the control strain (M401) 1 month after inoculation. This result is especially interesting because the plants in the field were watered in the same way as crops would have been in an agricultural setting. In the following months, however, the proportion of nodules occupied by M403 did not change significantly, whereas that of nodules occupied by M401 increased, reaching levels similar to those for M403 2 months later. Once this level of nodule occupancy was attained by M401, it remained constant throughout the rest of the experiment. These results imply that an equilibrium in nodule occupancy was reached by both types of inoculum but that this equilibrium was reached more rapidly by M403. We cannot rule out that the transient advantage in competitiveness could be due to the reduced plasmid stability observed in M403 (see above). The integration of the modified *putA* gene into the chromosome would clarify this question.

Studies similar to those described in this work were performed by Robleto et al. (24) with *R. etli* strains. Those authors sought to determine whether trifolitoxin production in these strains increased nodulation competitiveness in the field. They showed that, over 2 years, the frequency of nodule occupancy by trifolitoxin-producing strains increased by at least 20%, even if these strains were coinoculated, in unfavorable proportions, with trifolitoxin-susceptible strains. Our results demonstrate that increasing *putA* gene expression increases competitiveness, although the increase is transitory. However, the mode by which competitiveness is increased is different because the competitive advantage conferred by modified *putA* gene expression is metabolic in nature, whereas trifolitoxin acts as an antibiotic. Trifolitoxin inhibits many α -proteobacteria (33), and trifolitoxin-producing strains have been shown to

have a significant impact on this group of microorganisms in the rhizosphere (25). Strains with modified *putA* gene expression should have a much less dramatic effect, and this is currently being investigated.

Our results suggest that strains combining a modified expression of the *putA* gene with a higher efficiency of nitrogen fixation could be of commercial value for use as an inoculum for alfalfa. Our results also suggest that these inoculants may be especially useful in conditions in which the plant suffers drought stress. Moreover, as many crops liberate proline and metabolic precursors in their root exudates, modified *putA* gene expression could potentially be used to increase rhizosphere colonization by other beneficial soil microorganisms in adverse conditions. Additional field releases under different climatic conditions may be useful to test the hypothesis that enhanced proline utilization can be strategy for improvement of inocula.

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