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Greenhouse and Field Evaluations of an Autoselective System Based on an Essential Thymidylate Synthase Gene for Improved Maintenance of Plasmid Vectors in Modified *Rhizobium meliloti*

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The stability of the *thy* autoselective system, based on an essential thymidylate synthase gene, for enhanced maintenance of plasmid vectors in *Rhizobium meliloti* was evaluated in the greenhouse and with field-grown alfalfa. The *thy* autoselective system consists of a free-replicating, broad-host-range plasmid vector containing a copy of the *thyA* gene from *Lactococcus lactis* subsp. *lactis* and a spontaneous mutant of *R. meliloti* deficient in thymidylate synthase (Thy$^-$). Under greenhouse conditions, Thy$^-$ rhizobia did not persist in rooting solution alone unless supplemented with thymidine but survived in the presence of the host plant. Nodules formed on alfalfa plants grown in thymidine-free rooting solution and inoculated with Thy$^-$ rhizobia contained only Thy$^+$ revertants. In soil, Thy$^-$ rhizobia were compromised and failed to nodulate alfalfa. Thy$^-$ mutants containing a *thy* plasmid survived in the rhizosphere and nodulated alfalfa like the wild-type strain. The *thy* autoselective system was tested in the field with Thy$^-$ strain Rm24T and pPR602, a *thy* plasmid vector devoid of antibiotic resistance genes and marked with constitutively expressed *lacZY*. At 80 days after sowing, most rhizobia isolated from the nodules of field-grown alfalfa inoculated with Rm42T(pPR602) contained pPR602. The *thy* autoselective system proved useful to ensure maintenance of the plasmid vector under greenhouse and field conditions in *R. meliloti*.

Genetic engineering is a promising technology for the development of improved *Rhizobium* strains for inoculation of legumes (4, 6, 27, 30, 32). Genes of particular interest can be introduced into rhizobia on broad-host-range, mobilizable but non-self-transmissible plasmids as vectors. Use of plasmid vectors may be preferred to direct insertion of sequences into the genome because of their ease of manipulation and possible copy number effects on the levels of gene expression (33).

Natural plasmids are quite stable in *Rhizobium* spp. (35) and even difficult to purposely eliminate from the cell. Protocols to facilitate the development of plasmid-cured derivatives of rhizobia have been proposed (1, 15), but curing of plasmids remains a challenging task (15, 21). On the other hand, maintenance and stability of exogenous natural plasmids or recombinant plasmids introduced into *Rhizobium meliloti* or other bacteria are frequently poor in the absence of selective pressure (8, 10, 17, 18, 27, 32). It is thought that plasmids that do not contribute to the fitness of the host can become lost from a population because the metabolic cost of their maintenance may decrease the specific growth rate of cells (14). In microorganisms introduced into soil, such plasmids are readily lost (26, 33).

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Therefore, maintenance of the plasmid vector in the heterologous host becomes a major consideration when the release of modified bacteria in the environment is planned. One solution to improve plasmid maintenance is to combine a host bacterium which has a mutation in a vital gene with a plasmid vector bearing a copy of that gene. Such a vector-host combination should be intrinsically stable because cells that would lose the plasmid would be compromised. This approach has been used in our laboratory for the construction of a *thy*-based vector system in *R. meliloti* (28, 29). Thymidylate synthase (EC 2.1.1.45) is a key enzyme for de novo DNA synthesis because it converts dUMP to dTMP by reductive methylation. This enzyme is essential for growth of certain microorganisms in the absence of an external supply of thymidine or thymine (7, 23). These two compounds are present at low concentrations in the environment, and survival of microorganisms deficient in thymidylate synthase is impaired. Strains of *R. meliloti* deficient in thymidylate synthase are readily obtained with media containing thymidine and an antifolate drug such as aminopterin or trimethoprim to inhibit dihydrofolate reductase (25, 29). The maintenance of a plasmid carrying a copy of the *Lactococcus lactis* subsp. *lactis thyA* gene, which codes for thymidylate synthase, is favored in a Thy$^-$ host, since only the cells containing such a plasmid are viable. Derivatives pPR601 and pPR602 of IncP plasmid vector pRK290 have been developed for that purpose (29).

The *thy*-based autoselective system leads to almost 100% plasmid maintenance in laboratory cultures of rhizobia (data not shown). The objective of the present work was to evaluate the *thy* vector system in *R. meliloti* under greenhouse and field conditions. Greenhouse testing is recognized as an important step in the evaluation of modified microorganisms prior to field release (30). This work was undertaken with a view to

TABLE 1. Description of bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source and/or reference
R. meliloti		
Rm41	Wild type	T. Sik (16)
Rm42	Sm^r mutant of Rm41	This work
Rm42(pPR602)		This work
Rm42T	Thy^- mutant of Rm42	This work
Rm42T(pPR602)		This work
CM2	Rif^r mutant of Rm41	Manian and O'Gara (19)
CM2(pGD500)		Ross et al. (29)
CM2(pPR601)		Ross et al. (29)
CM21	Thy^- mutant of CM2	Ross et al. (29)
CM21(pGD500)		This work
CM21(pPR601)		This work
GMI42	Wild type, Rif^r	P. Boistard (Institut National de la Recherche Agronomique, Toulouse, France)
E. coli		
HB101	Used as a host for helper pRK2013	Boyer and Roulland-Dussoix (5)
HX2	HB101 containing *thyA*	Ross et al. (28)
Plasmids		
pRK290	Broad-host-range vector; Tc^r	Ditta et al. (9)
pGD500	*lacZY* promoter probe derivative of pRK290; Tc^r	Ditta et al. (8)
pPR601	*thyA lacZY* Tc^r; derivative of pGD500	Ross et al. (29)
pPR602	*thyA lacZY*; derivative of pPR601	Ross et al. (29)
pRK2013	Helper plasmid for mobilization; Km^r	Figurski and Helinski (13)

future use of the vector system for introduction of various genes of interest into rhizobial inoculant strains.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The objective of this study was to assess whether the maintenance of plasmid vector pGD500 could be improved in *R. meliloti* by using a *thy*-based autoselective system. Plasmid pGD500 is a derivative of the broad-host-range non-self-transmissible 20-kb vector pRK290 and confers tetracycline resistance to the host. Genes *thyA* from *L. lactis* subsp. *lactis* and *lacZY* from *Escherichia coli* have been cloned into pGD500, resulting in pPR601 (29). The *thyA* gene from *L. lactis* was chosen to limit possible repair of the mutated *thy* gene in *R. meliloti* by homologous recombination. Plasmid pPR602 (*lacZY thyA*) is a derivative of pPR601 devoid of antibiotic resistance genes (29). All strains and plasmids used in this work are listed in Table 1.

E. coli was grown in Luria-Bertani (20) broth at 37°C with shaking. Rhizobia were grown in minimal salt medium (24) containing 0.4% mannitol as a carbon source and 0.1% glutamate (i.e., MSG medium) or 0.1% yeast extract (i.e., MSY medium) as a nitrogen source. Rhizobial cultures were incubated at 30°C with shaking. Thymidine (50 μg/ml) was added to grow Thy^- mutants. Antibiotics were used at the following concentrations: rifampin, 100 μg/ml; tetracycline, 10 μg/ml; and streptomycin, 150 μg/ml.

Rm42(pPR602) was maintained on MSG medium supplemented with the chromogenic compound 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Plasmid pPR602 does not carry any selective marker, and Rm42(pPR602) was subcultured before use to verify the presence of pPR602. The other constructs were maintained on MSG medium.

Genetic manipulations. Construction of a *thy*-based vector system for *R. meliloti* included the development of Thy^- rhizobial mutants and the mobilization of a *thyA*-containing plasmid vector into the mutants. Some of the Thy^- mutants and complemented rhizobia have been obtained previously (29). Genetic manipulations were carried out as follows.

A Sm^r derivative of wild-type strain Rm41, Rm42, was selected. A spontaneous Thy^- derivative of Rm42, Rm42T, was obtained in the presence of 200 μg of aminopterin per ml. The mutation was stable in Rm42T in vitro, as indicated by the lack of reversion of the Thy^- phenotype in the presence of thymidine (data not shown). Rm42T requires thymidine for growth, even on complex laboratory media such as MSY and Luria-Bertani media. CM21 is another spontaneous Thy^- mutant and was derived from CM2, which is a spontaneous Rif^r mutant of Rm41 (11, 29).

Plasmids were mobilized from *E. coli* HX2 into *R. meliloti* in triparental matings with helper plasmid pRK2013 present in *E. coli* HB101. Plasmids pPR601 and pGD500 were each introduced into strain CM21. Plasmid pPR602 was mobilized into Rm42 and Rm42T. When Rm42T was used as recipient, exconjugants were selected on MSG plates. Exconjugant Rm42(pPR602) was selected on the basis of colony phenotype on MSG medium supplemented with X-Gal. On that medium, exconjugants appeared after 3 or 4 days of incubation at 28°C as mucoid vigorously growing blue colonies.

Evaluation of Thy^- rhizobial mutants in gnotobiotic rooting solution. The effect of the Thy^- mutation on the fitness of *R. meliloti* was studied by comparing strain CM2 and its Thy^- mutant CM21 for survival in gnotobiotic rooting solution in the absence of plant roots and in the presence of sugar beet or alfalfa. Rooting solution was chosen because it allowed the experiment to be carried out with and without the addition of thymidine (25 μg/ml). Rhizobia were grown as described above and were washed three times with 1/4-strength Ringer solution (Oxoid). Seeds of *Medicago sativa* cv. Europe and *Beta vulgaris* cv. Rex were surface sterilized and germinated as previously described (34). Two-day-old seedlings were transferred into large test tubes (one seedling per tube) containing a filter-paper support soaking in 10 ml of a carbon-free nitrogen-free rooting solution (34). Seedlings were inoculated in the tubes with 0.1-ml suspensions of rhizobial cells (i.e., about 10^7 CFU). A total of 43 plants were used per treatment as follows: three plants were used to assess rhizosphere colonization, and the other determinations were performed on 40 plants (i.e., 20 plants in duplicate). The tubes without seedlings also received 0.1-ml volumes of the cell suspensions. The tubes were put in a greenhouse with a 16-h day length (tungsten lighting; 10,000 lux; minimal temperature, 14°C; maximal temperature, 22°C).

Sampling for enumeration of rhizobia was carried out 28 days after inoculation. Three tubes without plants were assessed by colony counts, using MSY plates supplemented with thymidine and rifampin. When plants were used, whole root systems (three plants) were removed from the rooting solution and each was vortexed for 7 min in 1/4-strength Ringer solution. Extracts and dilutions were plated onto MSY plates supplemented with thymidine and rifampin. In each CM21 treatment, 20 isolated colonies were chosen at random per replicate and screened for Thy phenotype by replica plating on MSY and MSY plates plus thymidine.

The number of nodules on alfalfa was monitored throughout the experiment, using 20 plants per replicate per treatment. The extent of nodulation (percentage) was calculated by reference to the final number of nodules present on alfalfa inoculated with Thy^+ wild-type CM2 in the absence of thymidine. In each replicate of the CM21 treatments, 20 nodules were collected at 28 days after inoculation (one nodule per plant) and were surface sterilized with methanol (30 s) and 20% (vol/vol) H_2O_2 (2 min). They were washed three times in sterile distilled water. The nodules were crushed in cold buffer, as described by Birkenhead et al. (2), and suspensions were streaked onto MSY agar supplemented with thymidine. Twenty rhizobial colonies were chosen at random from each nodule, and the Thy phenotype of each colony was ascertained.

Evaluation of the *thy* autoselective system in soil microcosms. Construct

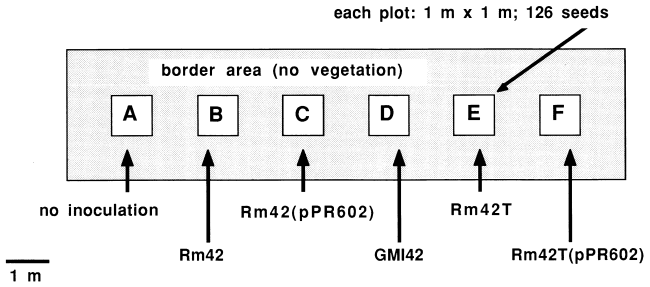

FIG. 1. Experimental design for the field release experiment conducted at the Fota site.

CM21(pPR601) was compared with CM2, CM21, and CM21(pGD500) for colonization and nodulation of alfalfa roots in soil microcosms. Soil material was collected at the Fota site, where the field release was to be carried out. The soil at the site is classified as a brown podzolic soil. The surface horizon (clay loam; pH_{H_2O} 7.1) was sampled (31), and the soil material was processed as described elsewhere (12). The soil was free of indigenous rhizobia capable of nodulating alfalfa (data not shown). Seeds were prepared as described above and were inoculated by dipping them into rhizobial suspensions (ca. 5×10^8 CFU/ml). Five seedlings were transferred into each 7.5-cm-diameter pot. Fifteen pots were used per treatment. The water content of the soil was adjusted to 60% saturation of the soil porosity with distilled water. Greenhouse conditions were as described above. For each treatment, three plants from one pot were used at each sampling time to monitor root colonization, as described above. The other pots were used after 28 days to determine nodule occupancy. A fifth treatment, i.e., inoculation with CM2(pPR601), was also studied. Five pots in duplicate were used per treatment, and four plants were sampled per pot. One nodule was chosen from each plant. Nodule occupancy was determined as in the previous experiment. Rif^r and Tc^r were used as markers for rhizobia and plasmids, respectively.

Statistical analyses. The greenhouse experiments were run in duplicate or triplicate. Analyses of variance were conducted on $\log_{10}$-transformed data for colony counts. Results were analyzed at $P = 0.05$ level.

Release site, field preparation, and experimental design. For field release, we constructed a *thy*-based vector system which consisted of a Sm^r Thy^- *Rhizobium* mutant (Rm42T) complemented with *thyA*-containing vector pPR602. Resistance to streptomycin was preferred over resistance to rifampin as the rhizobial marker because there was a lower background level of the former phenotype in the indigenous microbiota capable of growing on MSY medium. Exogenous antibiotic resistance genes should be avoided when designing genetically modified microorganisms (GMMs) for field release (30). Plasmid pPR602 confers $LacZ^+$ phenotype to rhizobial hosts but is free of antibiotic resistance genes and was chosen instead of pPR601. Release was carried out at the Fota estate located on Fota Island, on the east bank of Cork Harbor. The site was the property of University College Cork and was part of a grass-based pasture.

An area (75 by 50 m^2) was tilled and bordered with a 1-m-high wire-gauze fence to prevent access to small animals. A 0.6-m-deep plastic liner was inserted in the soil to limit lateral movement of microorganisms. An area (3 by 13 m^2) was marked as the actual release site. Six 1-m^2 plots in a straight line, separated from each other and from the rest of the site by a 1-m border area, were designated for the different experimental treatments.

Six plots, corresponding to six treatments, were used (Fig. 1). The treatments were not replicated. Plot A was sown with seeds that were not inoculated. Plots B to F were sown with seeds inoculated with Rm42, Rm42(pPR602), GMI42, Rm42T, and Rm42T(pPR602), respectively. GMI42 is a commercial strain of *R. meliloti* not related to Rm42 and was used as a commercial control.

Inoculation and planting. Rhizobia for inoculants were grown in 800-ml batches of MSG medium (supplemented with thymidine for strain Rm42T). Mid-log cultures were washed twice in 1/4-strength Ringer solution, and the cells were resuspended in 100 ml of the same solution prior to use (on the same day).

M. sativa L. cv. Europe is effectively nodulated by strains Rm41 and Rm42 under greenhouse conditions (data not shown) and was used in the field. The seeds were pelleted with a proprietary coating, courtesy of Irish Sugar plc (Carlow, Ireland). A total of 4 g of coated seeds (ca. 200 seeds) was then inoculated on site with 1 ml of rhizobial suspension prior to sowing.

Each plot received 126 seeds. Seeds were planted along six parallel rows, with a distance of 14 cm between rows. Each row received 21 seeds, which were spaced 4.5 cm apart. The remainder of the rhizobial suspensions (99 of 100 ml) was equally dispensed over the 1-m^2 plots. This resulted in a level of inoculum of about 5×10^{10} rhizobia per m^2 for the inoculated treatments. The furrows were covered by 1 cm of soil.

Harvesting, ARA, and nodule occupancy. At 80 days after sowing, 15 alfalfa plants were removed from each plot and transported to the laboratory for further analysis. The nodules from 10 of these plants were collected and pooled for the acetylene reduction assay (ARA), which was carried out as described by Bolton et al. (3).

All nodules from five plants were used to isolate rhizobia, as described above. Rhizobia were isolated on MSY agar supplemented with thymidine. Twenty colonies were chosen per nodule. The colonies were studied for Thy phenotype and for resistance to rifampin (marker for GMI42) and to streptomycin and were screened on X-Gal-containing medium for LacZ phenotype determination.

Regulatory aspects for the release of GMMs. Recombinant rhizobia are usually considered as posing a low risk to human health and the environment (30). The rhizobial strains containing plasmid pPR602 correspond to GMMs, and permission for their deliberate release in the environment was requested from the Irish National rDNA Committee (29 January 1990), which evaluates applications for release of GMMs on a case-by-case basis by using guidelines intended to protect plants, animals, and the human environment. As part of the evaluation, information was requested by the Committee on a format inspired from the proposed European Community directive on release of GMMs. The latter (directive 90/220/EC) was adopted on 23 April 1990 and was implemented in Ireland at a date subsequent to this field release (22). Official authorization was granted by the Committee on 10 December 1990. Sowing took place on 26 July 1991, and harvesting took place on 15 October 1991. This field release of GMMs was the first one carried out in Ireland.

RESULTS

Influence of thymidine availability on survival of a Thy^- *Rhizobium* mutant in gnotobiotic rooting solution. Survival of Rif^r-marked Thy^+ wild-type CM2 and its Thy^- mutant CM21 in rooting solution was monitored during a 28-day period (Table 2). In rooting solution alone, strain CM2 was recovered at 28 days but CM21 survived only when thymidine had been added to the rooting solution. In the latter case, all rhizobia recovered were Thy^-, as indicated by the lack of growth on solid MSG medium. The experiment was also carried out in the presence of sugar beet seedlings whose roots were immersed in the rooting solution. Essentially similar results were obtained (Table 2). However, when the experiment was run in the presence of alfalfa, rhizobia were recovered from the CM21 treatment even when thymidine was not added to the rooting solution (Table 2). In this latter case, CM21 rhizobia were found at a lower population level at 28 days and further analysis showed that 27% of them were rhizobia that had reverted to the Thy^+ phenotype of the wild-type CM2.

Influence of thymidine availability on nodulation of alfalfa by a Thy^- *Rhizobium* mutant in gnotobiotic rooting solution. Thy^- mutant CM21 was inferior to Thy^+ strain CM2 for nodulation of alfalfa grown in rooting solution (Table 3). When no thymidine was provided in the rooting solution, nodulation by

TABLE 2. Influence of exogenous supply of thymidine on survival of Thy^+ strain CM2 and Thy^- mutant CM21 and stability of Thy^- phenotype when rhizobia were inoculated into gnotobiotic rooting solution alone or rooting solution growing sugar beet or alfalfa

Treatment[a]		Rhizobial density[b]		
Rhizobial strain	Thymidine	No plant	Sugar beet	Alfalfa
CM2	None added	4.5 a	6.4 a	6.7 a
	Added	4.6 a	6.2 a	6.8 a
CM21	None added	0 b	0 b	5.9 b (73 a)
	Added	4.5 a (100)	6.8 a (100)	6.9 a (100 b)

[a] Rhizobia were inoculated at the level of 6 log CFU per ml of rooting solution. Thymidine was added at 25 μg/ml of rooting solution.

[b] Rhizobial numbers at 28 days after inoculation are expressed as log CFU per milliliter of rooting solution when no plant was present; otherwise rhizobial numbers are expressed as log CFU per root system. The detection limit was 1 log CFU per ml of rooting solution (when no plant was present) or 2 log CFU per root system (sugar beet or alfalfa). Letters (in roman) are used to indicate statistical differences between treatments. The percentage of rhizobia displaying a Thy^- phenotype is given in parentheses. All rhizobia originating from the same nodule displayed the same Thy phenotype.

TABLE 3. Influence of exogenous supply of thymidine in rooting solution on nodulation of alfalfa by Thy$^+$ strain CM2 and Thy$^-$ mutant CM21 and stability of Thy$^-$ phenotype in CM21 rhizobia isolated from nodules

Treatment[a]		Alfalfa[b]		
Rhizobial strain	Thymidine	Extent of nodulation (%) on day 28[c]	Time (days) for 50% of day 28 nodulation	Rhizobia with a Thy$^-$ phenotype (%)
CM2	None added	100 a	14 a	NA[d]
	Added	100 a	12 a	NA
CM21	None added	69 b	23 b	0 a
	Added	71 b	15 a	100 b

[a] Rhizobia were inoculated at the level of 6 log CFU per ml of rooting solution. Thymidine was added at 25 μg/ml of rooting solution.
[b] Letters (in roman) are used to indicate statistical differences between treatments.
[c] The extent of nodulation was calculated by reference to the number of nodules in the CM2 treatment when no thymidine was added.
[d] NA, not applicable.

TABLE 4. Nitrogenase activity (ARA) and maintenance of *thy* plasmid pPR602 in nodules of alfalfa sampled at the Fota field experiment

Introduced rhizobial strain	ARA (nmol of acetylene/g of fresh nodule/min)	Plasmid maintenance (%; LacZ phenotype)		
		LacZ$^+$	LacZ$^-$	Mixed[a]
None	NA[b]	NA	NA	NA
Rm42	377	NA	NA	NA
Rm42(pPR602)	82	67	26	7
Rm42T	67	NA	NA	NA
Rm42T(pPR602)	146	91	6[c]	3[d]

[a] Nodules with mixed occupancy contained both LacZ$^+$ and LacZ$^-$ rhizobia.
[b] NA, not applicable.
[c] 6% corresponds to two nodules that were derived from the same plant, and every colony recovered was able to grow on MSG medium without the addition of thymidine.
[d] 3% corresponds to one nodule, which yielded 1 LacZ$^-$ colony of 12.

CM21 was delayed and all rhizobia recovered from nodules were Thy$^+$ revertants (Table 3). When thymidine was added, nodulation occurred as early as in the CM2 treatments and no Thy$^+$ revertants were found in the nodules formed by CM21.

Survival of a Thy$^-$ *Rhizobium* mutant and maintenance of a *thy*-containing plasmid vector in the rhizosphere of soil-grown alfalfa. In greenhouse soil microcosms, Thy$^+$ strain CM2 was recovered at about 5 log CFU per root at 28 days after inoculation (Fig. 2). The Thy$^-$ mutant CM21 declined rapidly in the rhizosphere of alfalfa and was recovered at about 2 log CFU per root at 28 days after inoculation (Fig. 2). All 20 rhizobial colonies recovered at each sampling time from the CM21 treatment on plates amended with rifampin and thymidine were unable to grow on MSG plates unless the medium was supplemented with thymidine.

Construct CM21(pGD500) displayed a Thy$^-$ phenotype and survived poorly, like strain CM21, in the rhizosphere of alfalfa (Fig. 2). Introduction of the heterologous *L. lactis* subsp. *lactis thyA* gene on a self-replicating plasmid (pPR601) into CM21 restored the Thy$^+$ phenotype of wild-type CM2 (29) and enabled the strain to survive at high cell densities in the rhizosphere of soil-grown alfalfa (Fig. 2).

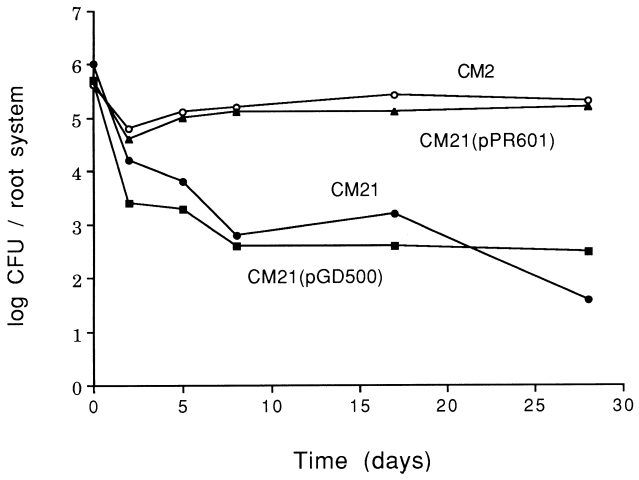

FIG. 2. Survival of Thy$^+$ wild-type CM2, Thy$^-$ mutant CM21, and CM21 containing vector pGD500 or its *thy* derivative pPR601 in the rhizosphere of alfalfa grown in soil microcosms for 28 days. Statistical analysis of data indicated that CM2 and CM21(pPR601) persisted at similar levels, whereas CM21 and CM21(pGD500) survived at comparable low cell densities.

Nodulation of soil-grown alfalfa by a Thy$^-$ *Rhizobium* mutant and *thyA*-complemented rhizobia. Unlike wild-type CM2, the Thy$^-$ mutant CM21 and CM21(pGD500) did not nodulate alfalfa in greenhouse soil microcosms. Introduction of heterologous *L. lactis* subsp. *lactis thyA* gene on a self-replicating plasmid into CM21 restored the Thy$^+$ phenotype of wild-type CM2 and its nodulation ability (data not shown). Investigation of rhizobia isolated from nodules indicated that *thyA* plasmid pPR601 was maintained at 87 and 98% in the wild-type CM2 and the Thy$^-$ mutant CM21, respectively, as indicated by the Tc^r phenotype of the colonies. All rhizobia isolated from nodules of alfalfa inoculated with CM21(pPR601) displayed a Thy$^+$ phenotype.

Nodulation of alfalfa and nitrogenase activity in the field. No nodules were present on alfalfa harvested from the control plot, confirming that Fota soil is free from indigenous *R. meliloti*. Nodules were found in each inoculated treatment, including on roots of plants inoculated with Thy$^-$ mutant Rm42T. ARA was carried out as a qualitative indication of symbiotic effectiveness. Results indicated that nitrogen fixation was effective for each inoculated treatment (Table 4). Dry shoot weight determinations were carried out. Results for the uninoculated control indicates that the Fota soil was not nitrogen limited (data not shown).

Nodule occupancy and stability of Thy$^-$ mutation in the field. A total of 60 nodules were sampled from plot E inoculated with Thy$^-$ mutant Rm42T, and rhizobia were obtained from 53 of these nodules. Most nodules (44 of 53) contained only Sm^r Thy$^+$ rhizobia that were assumed to be revertants because alfalfa plants grown in the uninoculated control plot were not nodulated. Only one nodule was occupied by the Thy$^-$ mutant strain alone. Another eight nodules displayed mixed occupancy of mostly Thy$^+$ revertants and a few Thy$^-$ rhizobia.

Maintenance of *thy* plasmid vector pPR602 in field-grown alfalfa. Plants were sampled from plots C and F previously inoculated with Rm42(pPR602) and Rm42T(pPR602), respectively. Rhizobia were isolated from nodules and screened for resistance to streptomycin for the ability to grow in the absence of thymidine, and for the LacZ phenotype. A total of 43 nodules were studied for plot C. All rhizobia were Sm^r. Of 43

nodules, 29 (i.e., 67%) were occupied by rhizobia containing *thyA lacZY* plasmid vector pPR602. The other nodules contained LacZ$^-$ rhizobia, indicating loss of pPR602, or displayed mixed occupancy (Table 4).

In comparison, 91% of nodules from alfalfa inoculated with Rm42T(pPR602) contained Sm^r rhizobia which contained pPR602, with another 3% of nodules containing both rhizobia with and without pPR602 (Table 4). Each LacZ$^-$ colony studied also displayed a Thy$^+$ phenotype, indicating that they were revertants.

DISCUSSION

The *thy* autoselective system proposed by Ross et al. (29) and evaluated in this report for improved maintenance of plasmid vectors in *R. meliloti* is based on the assumption that survival of Thy$^-$ rhizobial mutants is compromised in the environment. Results obtained in gnotobiotic rooting solution and in soil microcosms substantiate this assumption. For instance, Thy$^-$ rhizobia failed to survive in rooting solution in the absence of an exogenous supply of thymidine (Table 2). In the presence of alfalfa, however, Thy$^-$ rhizobia were able to persist, indicating that the host plant could supply some thymine or thymidine as root exudates. In contrast, the non-host plant sugar beet did not sustain survival of the mutants. Another assumption regarding the *thy* system is that the Thy$^-$ mutation should be stable. Stability of the Thy$^-$ phenotype in rhizobial mutants was monitored. Revertants were not recovered in rooting solution supplemented with thymidine, but some Thy$^+$ revertants were found at the root surface of alfalfa grown in rooting solution not supplemented with thymidine (Table 2). In the latter experiment, all rhizobia isolated from nodules were Thy$^+$, and in the field, the majority of bacteroids in the Thy$^-$ treatment were presumably revertants. Clearly, instability of the Thy$^-$ mutation selected following a spontaneous event (29) is a limiting factor of this particular *thy* system under certain circumstances.

The *thy* autoselective system consists of a Thy$^-$ strain and a plasmid vector bearing a copy of the *thyA* gene. The requirement for thymidine constitutes a positive selection pressure that is likely to favor vector-containing cells. Indeed, the presence of a *thy* plasmid increased the ecological fitness of Thy$^-$ rhizobial mutants, and such *thy* plasmid vectors were maintained at a higher rate in Thy$^-$ rhizobial mutants than in Thy$^+$ wild-type strains. For instance, the *thy* plasmid vector pPR601 was maintained at a higher rate in the Thy$^-$ rhizobial mutant CM21 than in the wild-type CM2 (98 versus 87%) in nodules of alfalfa grown in soil microcosms. In the laboratory and under greenhouse conditions, the *thy* system ensured stable plasmid maintenance during symbiosis. Maintenance of *thy* plasmid vector pPR602 was also high (91%) in rhizobia isolated from nodules of field-grown alfalfa previously inoculated with Rm42T(pPR602) (Table 4), but the spontaneous Rm42T mutant strain apparently reverts to a wild-type Thy$^+$ phenotype at rather high frequency.

Plant yield obtained from the uninoculated control in the field release experiment indicates that the Fota soil was not nitrogen limited (data not shown). Although treatments were not replicated, large differences in nitrogenase activity between treatments suggest that the presence of *thy* plasmid vector pPR602 might affect N_2 fixation by Thy$^+$ Rm42 (Table 4). Some engineered plasmids introduced into other strains of *R. meliloti* had a negative effect on the effectiveness of the strain (27). However, we never observed similar effects with closely related plasmids. The presence of this cloned *thyA* gene may influence symbiotic efficiency in *R. meliloti*. This issue deserves further investigation.

In conclusion, the *thy* vector system is effective in assuring stable plasmid maintenance in inoculant strains of *R. meliloti* under field conditions. Work is currently in progress to construct a stable rhizobial Thy$^-$ mutant strain by reverse genetics to further improve plasmid maintenance in the *thy* autoselective system. Regulated expression of the *thy* gene may be required to overcome any negative effect that the presence of plasmid-borne *thyA* gene might have on symbiotic effectiveness. The *R. meliloti thy* gene has been cloned by complementation. This clone will allow analysis and genetic manipulation of the *thy* locus of *R. meliloti* to be undertaken.

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