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Hydrogen Evolution from Alfalfa and Clover Nodules and Hydrogen Uptake by Free-Living *Rhizobium meliloti*†

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A series of *Rhizobium meliloti* and *Rhizobium trifolii* strains were used as inocula for alfalfa and clover, respectively, grown under bacteriologically controlled conditions. Replicate samples of nodules formed by each strain were assayed for rates of H_2 evolution in air, rates of H_2 evolution under Ar and O_2, and rates of C_2H_2 reduction. Nodules formed by all strains of *R. meliloti* and *R. trifolii* on their respective hosts lost at least 17% of the electron flow through nitrogenase as evolved H_2. The mean loss from alfalfa nodules formed by 19 *R. meliloti* strains was 25%, and the mean loss from clover nodules formed by seven *R. trifolii* strains was 35%. *R. meliloti* and *R. trifolii* strains also were cultured under conditions that were previously established for derepression of hydrogenase synthesis. Only strains 102F65 and 102F51 of *R. meliloti* showed measurable activity under free-living conditions. Bacteroids from nodules formed by the two strains showing hydrogenase activity under free-living conditions also oxidized H_2 at low rates. The specific activity of hydrogenase in bacteroids formed by either strain 102F65 or strain 102F51 of *R. meliloti* was less than 0.1% of the specific activity of the hydrogenase system in bacteroids formed by H_2 uptake-positive *Rhizobium japonicum* USDA 110, which has been investigated previously. *R. meliloti* and *R. trifolii* strains tested possessed insufficient hydrogenase to recycle a substantial proportion of the H_2 evolved from the nitrogenase reaction in nodules of their hosts. Additional research is needed, therefore, to develop strains of *R. meliloti* and *R. trifolii* that possess an adequate H_2-recycling system.

The evolution of H_2 from soybean root nodules was first reported by Hoch et al. (10). Additional research (11) provided evidence that H_2 from nodules was produced during the nitrogenase reaction. Loss of H_2 from nodules of cowpeas has also been observed (4), but the potential agricultural significance of energy loss from nodules through H_2 evolution was not appreciated until Schubert and Evans (16) reported that the extent of loss in many nodulated legumes ranged from 40 to 60% of the energy flux through the nitrogenase system. H_2 evolution via nitrogenase requires adenosine triphosphate and reductant (2) and therefore has been considered a source of inefficiency in the N_2 fixation process (16, 17). The supply of energy as photosynthate to nodules has been reported to be a major factor limiting N_2 fixation in legumes (9). Under conditions where energy is limiting, the magnitude of H_2 loss would be expected to influence the quantity of N_2 fixed by the plant.

Nodules from several species of nonleguminous plants (16), pea nodules formed by strain ONA 311 of *Rhizobium leguminosarum* (5), and nodules of cowpeas and soybeans formed by a few selected strains of *Rhizobium* (3, 15, 16) evolved no H_2 in air. An H_2 uptake system could be demonstrated in all nodules that evolved no H_2 under a normal atmosphere of air. The hydrogenase system in bacteroids is known to participate in the recycling of the H_2 produced by the nitrogenase reaction (3, 5, 6, 14, 17). The oxidation of externally supplied H_2 by H_2 uptake-positive bacteroids has been shown to generate adenosine triphosphate (6, 7), protect nitrogenase from O_2 damage (7), and provide a mechanism for the conservation of carbon substrates (12). Hydrogenase systems in *Azotobacter chroococcum* (20) and certain blue-green algae (1, 21) also have been identified that function in an H_2-recycling process.

Greenhouse and field experiments have indicated a beneficial effect of the H_2 uptake system in nodules on the N_2 fixation process (18; H. J. Evans, D. W. Emerich, T. Ruiz-Argüeso, R. J. Maier, and S. L. Albrecht, *in Proceedings of the Steenbock-Kettering International Symposium on Nitrogen Fixation*, in press). Since a rela-

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tively few strains of *Rhizobium* are known with a capacity for producing nodules that recycle H_2, screening of additional strains for the presence of hydrogenase is needed. We have estimated relative efficiencies (17) of nitrogen fixation by alfalfa and clover nodules formed by a series of strains of *Rhizobium meliloti* and *Rhizobium trifolii*, respectively. This provides an index which estimates the proportion of the electron flux through nitrogenase that is utilized for N_2 reduction. Also, the capacity of a series of strains of *Rhizobium* to synthesize hydrogenase under free-living conditions has been determined.

MATERIALS AND METHODS

Cultures. Strains of *R. meliloti* and *R. trifolii* were obtained from sources listed in Table 1, with the exception of strain OSUT2, which was isolated from alsike clover (*Trifolium hybridum*) plants from a field near Corvallis, Ore. Cultures were maintained on slants of a yeast extract-mannitol agar (22) and stored at 4°C.

Plant inoculation and growth. Seeds of white clover (*Trifolium repens* cv. 'New Zealand') and alfalfa (*Medicago sativa* cv. 'Vernal') were surface disinfected (22) and germinated on plates containing 1% agar. Young seedlings were planted in a 1:1 sand-vermiculite mixture in autoclaved Leonard jar assemblies containing one-fifth-strength Jensen nutrient solution (22). At 3 days after planting, 10 ml of yeast extract-mannitol broth cultures of *R. meliloti* or *R. trifolii* (3 to 5 days old with optical densities near 1.0) was pipetted into each Leonard jar unit at the base of the seedlings. Plants were grown in a greenhouse provided with supplemental light of 5,400 lx during a 15-h light period. The temperature for growth of white clover plants was maintained at 25°C during the day and 16°C at night; for alfalfa plants the temperature was 27 and 20°C for day and night, respectively. At 2 days before the assays were conducted, Leonard jar cultures of plants were transferred to a controlled environmental chamber with 21,500-lx illumination and photoperiod and temperature regimes that were the same as those used in the greenhouse.

TABLE 1. *Origin of Rhizobium strains used in experiments*

Species	Strain	Source
R. meliloti	102F45	J. C. Burton, Nitragin Co., Milwaukee, Wis.
R. meliloti	102F65	J. C. Burton
R. meliloti	102F34	J. C. Burton
R. meliloti	102F58	J. C. Burton
R. meliloti	102F32	J. C. Burton
R. meliloti	102F28	J. C. Burton
R. meliloti	102F51	J. C. Burton
R. meliloti	623/A	L. Barber, Oregon State University, Corvallis
R. meliloti	SU51	J. M. Vincent, University of Sydney, Sydney, Australia
R. meliloti	SU27	J. M. Vincent
R. meliloti	142	P. E. Bishop, North Carolina State University, Raleigh
R. meliloti	3Doa7	D. Weber, USDA, Beltsville, Md.
R. meliloti	3Doa8	D. Weber
R. meliloti	CP119	J. L. Marlow, Celpril Industries, Inc., Manteca, Calif.
R. meliloti	CP115	J. L. Marlow
R. meliloti	CP10	J. L. Marlow
R. meliloti	CP121	J. L. Marlow
R. meliloti	CP19	J. L. Marlow
R. meliloti	ATCC 10312	P. Wong, Kansas State University, Manhattan
R. trifolii	SU79Y	J. M. Vincent
R. trifolii	TA1	P. E. Bishop
R. trifolii	0403	P. E. Bishop
R. trifolii	J1	F. B. Dazzo, Michigan State University, East Lansing
R. trifolii	161x68	J. C. Burton
R. trifolii	T1	S. R. Russell, Oregon State University, Corvallis

Assays of nodules. Segments of roots (1 to 2 cm), each containing 0.05 to 0.2 g of fresh nodules, were excised, and C_2H_2 reduction assays were initiated within 1 min by using the method of Schwinghamer et al. (19). A separate sample of nodules was obtained, and H_2 evolution and O_2 consumption rates were measured by the amperometric method (16, 23; F. J. Hanus, K. R. Carter, and H. J. Evans, Methods Enzymol., in press). H_2 evolution rates also were determined in a gas mixture of 79.96% Ar, 20% O_2, and 0.04% CO_2. In this procedure two Clark-type electrodes (model 4004; Yellow Springs Instruments, Yellow Springs, Ohio) were maintained in a closed chamber constructed of Lucite. One of the electrodes was used to monitor O_2 uptake, and the other measured H_2 evolution.

The rates of H_2 evolution from nodules in air and the rates of C_2H_2 reduction were used to calculate the relative efficiencies of N_2 fixation by nodules as described by Schubert and Evans (16). The relative efficiency value = 1 − [(rate of H_2 evolution in air)/(rate of C_2H_2 reduction or rate of H_2 evolution in Ar-O_2)]. Relative efficiency values based upon rates of H_2 evolution in air and rates of H_2 evolution under Ar-O_2-CO_2 also were calculated, but they are meaningful only for samples which lack hydrogenase activity and do not recycle H_2. When relative efficiency values based on C_2H_2 reduction rates were greater than those based on rates of H_2 evolution under Ar-O_2-CO_2, the presence of hydrogenase and an H_2-recycling process was suspected.

Assay for hydrogenase in free-living rhizobia. Slants of a medium designed for growth of cells with hydrogenase activity (13) were made in test tubes (15 by 150 mm) and inoculated with *R. meliloti* and *R. trifolii* grown on a yeast extract-mannitol medium (22). After 4 days of incubation in air at 26°C, each test tube cap was replaced by a sterile serum stopper, and 1 ml of 10% H_2 in air was injected into each test tube, which was then incubated for an additional 10 h at 26°C. Gas samples (0.5 ml) were removed from each culture tube after 10 and 18 h of incubation and analyzed for H_2 by gas chromatography (13). The H_2 lost over an 8-h period was determined. Five replicate cultures were used per strain of *Rhizobium* tested. Uninoculated slants of the culture medium were used as controls to monitor leakage of H_2 from the test

tubes. Cells were harvested from each slant, and protein was determined by the method of Goa (8) by using procedures (13) to adapt the method for use with whole cells.

Preparation of bacteroids. Alfalfa seeds (cv. 'Vernal') were surface disinfected, germinated, planted, and grown as described above. After 30 days of growth, nodules were harvested, 3 g of nodules formed by each strain (*R. meliloti* 102F65 and 102F51) was crushed aerobically, and bacteroid suspensions were prepared from the crushed nodules by the methods used for the preparation of *Rhizobium japonicum* bacteroids (7). The final bacteroid pellets (approximately 150 and 174 mg [dry weight] of bacteroids for strains 102F65 and 102F51, respectively) were suspended in 6.0 ml of 0.05 M potassium phosphate buffer (pH 7.0) containing 2.5 mM $MgCl_2$ for measuring H_2 uptake activity by the amperometric method (16, 23; Hanus et al., in press).

RESULTS AND DISCUSSION

Results from an initial survey (16) indicated that commercial inocula for clover and alfalfa produced nodules that evolved relatively large amounts of H_2. In search of more efficient rhizobia, surveys were conducted of H_2 evolution and C_2H_2 reduction of nodules formed on alfalfa and clover by 19 strains of *R. meliloti* and 7 strains of *R. trifolii*, respectively. All of the rhizobial strains tested produced nodules which evolved H_2 in air (Tables 2 and 3). A representative amperometric recording of H_2 evolution by alfalfa nodules formed by strain SU51 is shown in Fig. 1. With this sample the rate of H_2 evolution in an atmosphere containing N_2 and O_2 was about one-fifth that observed when Ar was substituted for N_2 in the atmosphere over nodules. As expected, flushing the cuvette with a mixture of Ar and CO_2 caused H_2 evolution to cease, presumably because O_2 was needed to support the respiratory synthesis of adenosine triphosphate. The characteristics of the reaction were those expected for the adenosine triphosphate-dependent reduction of protons by the nitrogenase system.

The yields of inoculated alfalfa and clover plants and the rates of H_2 evolution and C_2H_2 reduction by nodules produced by groups of *R. meliloti* and *R. trifolii* strains are presented in decreasing order of nodule relative efficiencies (Tables 2 and 3). The relative efficiencies for alfalfa nodules formed by the *R. meliloti* strains ranged from 0.65 to 0.83, and the mean for the 19 strains tested was 0.75. For clover nodules the relative efficiency values ranged from 0.51 to 0.75, and the mean for the seven strains tested

TABLE 2. *Hydrogen losses and relative efficiencies of nitrogen fixation of nodules from alfalfa (M. sativa cv. 'Vernal') as affected by the strain of R. meliloti*[a]

Strain	C_2H_2 reduction (μmol/h per g of fresh nodules)	H_2 evolution (μmol/h per g of fresh nodules) in:		Relative efficiency[b]	Plant dry wt (g/culture)[c]
		Air	Ar-O_2-CO_2		
Expt 1					
SU51	24.0 ± 1.2	4.2 ± 0.6	21.8 ± 2.3	0.83 ± 0.02	3.08 ± 0.47
102F45	33.0 ± 4.4	5.5 ± 1.9	30.4 ± 9.3	0.82 ± 0.07	3.50 ± 0.44
102F65	31.7 ± 3.9	5.9 ± 0.6	23.9 ± 4.4	0.81 ± 0.03	2.84 ± 0.05
102F34	30.5 ± 2.1	6.7 ± 2.1	28.4 ± 4.1	0.78 ± 0.03	2.77 ± 0.05
102F51	28.7 ± 5.3	5.7 ± 0.4	26.9 ± 2.9	0.78 ± 0.03	3.57 ± 0.66
623/A	32.6 ± 0.8	5.9 ± 1.1	32.4 ± 9.0	0.78 ± 0.04	3.22 ± 0.99
SU27	6.2 ± 0.2	1.6 ± 0.3	5.6 ± 1.6	0.77 ± 0.09	0.60 ± 0.07
142	23.7 ± 4.4	5.9 ± 0.5	29.2 ± 2.5	0.76 ± 0.04	2.65 ± 0.41
102F58	37.8 ± 5.3	9.9 ± 1.5	40.5 ± 5.0	0.73 ± 0.06	3.25 ± 0.17
102F32	21.3 ± 1.8	6.6 ± 0.5	27.5 ± 2.3	0.69 ± 0.02	2.87 ± 0.12
102F28	21.7 ± 1.6	6.6 ± 1.3	24.2 ± 4.0	0.69 ± 0.08	2.09 ± 0.25
Expt 2					
ATCC 10312	21.8 ± 1.0	4.1 ± 0.5	17.4 ± 4.9	0.81 ± 0.02	2.23 ± 0.24
3Doa8	25.7 ± 6.2	6.2 ± 2.4	21.9 ± 5.6	0.78 ± 0.03	2.56 ± 0.33
CP119	27.1 ± 3.3	5.9 ± 0.5	25.7 ± 2.3	0.75 ± 0.06	4.50 ± 0.36
3Doa7	24.5 ± 2.1	6.6 ± 1.0	24.2 ± 2.2	0.73 ± 0.05	3.13 ± 0.26
CP19	20.4 ± 2.2	6.3 ± 1.2	21.4 ± 2.2	0.70 ± 0.04	1.43 ± 0.33
CP10	31.1 ± 2.8	9.5 ± 2.5	27.9 ± 5.6	0.69 ± 0.07	2.95 ± 0.50
CP121	28.3 ± 3.2	9.5 ± 1.5	28.7 ± 2.2	0.67 ± 0.04	2.51 ± 0.41
CP115	24.7 ± 1.9	8.7 ± 1.8	27.9 ± 3.6	0.65 ± 0.07	2.36 ± 0.23

[a] Data presented are means ± standard error of the mean of determinations made on four or five replicate cultures of six plants each after a growth period of 55 days.

[b] Relative efficiency = 1 − (rate of H_2 evolution in air/rate of C_2H_2 reduction).

[c] Uninoculated control plants were not nodulated and yielded a mean of 0.03 ± 0.001 g of plant dry weight per culture.

TABLE 3. *Hydrogen losses and efficiency of nitrogen fixation of nodules from white clover (T. repens cv. 'New Zealand') as affected by the strain of R. trifolii*[a]

Strain	C_2H_2 reduction (μmol/h per g of fresh nodules)	H_2 evolution (μmol/h per g of fresh nodules) in:		Relative efficiency[b]	Plant dry wt (g/culture)[c]
		Air	Ar-O_2-CO_2		
JI	65.2 ± 10.7	14.0 ± 1.6	40.4 ± 4.2	0.75 ± 0.03	5.80 ± 0.71
OSUT2	20.4 ± 6.9	5.9 ± 1.8	19.9 ± 5.5	0.70 ± 0.03	3.02 ± 0.48
0403	46.9 ± 4.3	21.3 ± 5.3	45.7 ± 4.8	0.69 ± 0.05	7.15 ± 1.30
162x68	36.1 ± 4.8	11.3 ± 1.7	39.4 ± 5.9	0.69 ± 0.01	2.50 ± 0.24
TA1	59.5 ± 10.8	19.2 ± 0.5	55.3 ± 10.8	0.64 ± 0.08	4.80 ± 1.76
T1	51.0 ± 5.0	21.9 ± 4.5	45.5 ± 8.6	0.58 ± 0.06	5.30 ± 1.46
SU794	42.7 ± 4.9	19.4 ± 2.5	55.6 ± 6.4	0.51 ± 0.10	2.97 ± 0.73

[a] Figures are means ± standard error of the mean of determinations made on four replicate cultures of six plants each after 55 days of growth.

[b] Relative efficiency = 1 − (rate of H_2 evolution in air/rate of C_2H_2 reduction).

[c] Uninoculated control plants were not nodulated and yielded a mean of 0.02 ± 0.01 g of plant dry weight per culture.

was 0.66 (Table 3). Assuming that C_2H_2 reduction rates represent the total electron flux through nitrogenase (16), a mean of 25% of the energy available to nitrogenase for all alfalfa nodules examined and a mean of 34% for all clover nodules tested were lost through H_2 evolution.

No striking differences were found between relative efficiencies calculated on the basis of rates of C_2H_2 reduction or rates of H_2 evolution under an atmosphere in which N_2 was replaced by Ar. Efficiencies based upon H_2 evolution under Ar-O_2-CO_2 therefore are not presented. The differences between rates of C_2H_2 reduction and rates of H_2 evolution under Ar-O_2-CO_2 for nodules formed by *R. meliloti* strains 102F65 and ATCC 10312 provide an indication that a hydrogenase system in these nodules may participate in the recycling of part of the H_2 evolved by nitrogenase (14). The relative efficiency values of nodules formed by these strains are among the highest in the experiments conducted (Tables 2 and 3).

Regression analyses in which plant dry weights in the three experiments (Tables 2 and 3) were plotted against nodule relative efficiencies showed no significant positive correlation of these parameters. Since nodules formed by strains of *R. meliloti* with the highest relative efficiencies of 0.80 to 0.83 (Table 2) lost H_2 at rates of 17 to 20% of the electron flux, it is clear that the level of hydrogenase within these nodules was very low and insufficient to recycle a major portion of the H_2 evolved from the nitrogenase reaction. About 25% of the electron flow would be expected to be lost as H_2 under conditions where no H_2 recycling occurred (Evans et al., in press). The results in Tables 2 and 3 may be compared with those obtained with soybeans (3), in which nodules without measurable

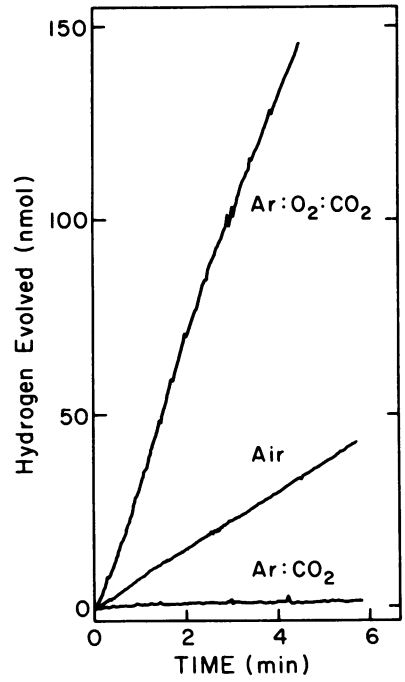

FIG. 1. *Continuous amperometric measurements of H_2 evolution from intact nodules of alfalfa inoculated with R. meliloti strain SU51. A sample of nodules (0.101 g, excised with small segments of roots) was placed in an electrode cuvette (2.8 ml), and H_2 evolution was followed continuously. The reaction was initiated in air and allowed to proceed for 6 min. The cuvette was then flushed for 30 s with 99.96% Ar-0.04% CO_2, and the rate was recorded (lower curve). In the reaction monitored in the upper curve, the cuvette with nodule segments was flushed for 30 s with a mixture of 79.96% Ar, 20% O_2, and 0.04% CO_2, and the reaction rate was recorded as shown. All three reaction rates were obtained with the same sample of nodules.*

hydrogenase lost H_2 to an extent of 25% or more of the nitrogenase electron flux, whereas nodules formed by *R. japonicum* strains with an active hydrogenase lost no measurable quantity of H_2. Since the presence of a hydrogenase with sufficient activity to recycle the major part of the H_2 produced by an efficient strain of *Rhizobium* seems to be a major factor affecting the efficiency of N_2 fixation, the failure to observe a significant positive correlation between nodule relative efficiency values and yields of clover or alfalfa in Tables 2 and 3 was not unexpected.

The mean ratio of moles of O_2 consumed per mole of C_2H_2 reduced by the alfalfa nodules formed by all 19 *R. meliloti* strains was 4.6 (range, 3.1 to 7.6). The corresponding mean ratio for clover nodules formed by seven strains of *R. trifolii* was 4.4, and the ratios ranged from 2.2 to 5.8. No positive correlation was observed between relative efficiency values and the ratios of moles of O_2 consumed per mole of C_2H_2 reduced.

Since the capacity to synthesize the hydrogenase system by free-living rhizobia has been demonstrated (13), *R. meliloti* strains with relative efficiencies of 0.73 or greater (Table 2) and the *R. trifolii* strains listed in Table 3 were assayed for asymbiotic H_2 uptake capacities. Two *R. meliloti* strains (102F65 and 102F51) took up H_2 at rates of 14.5 ± 5.2 and 7.4 ± 2.0 nmol of H_2 utilized per h per mg of protein, respectively, after they had been grown for 4 days on a medium designed for inducing hydrogenase (13). H_2 uptake activity in these two strains was not detected, however, unless cells were preincubated under H_2 before the assays. The induction by H_2 was observed also in studies of hydrogenase activity in free-living cultures of *R. japonicum* (13). None of the *R. trifolii* strains listed in Table 3 was capable of utilizing H_2 when cultured on the hydrogenase induction medium.

To confirm the presence of hydrogenase in nodules formed by the two *R. meliloti* strains capable of utilizing H_2 under free-living conditions, bacteroid suspensions were prepared from nodules formed on alfalfa by each of the two strains showing H_2 uptake under asymbiotic conditions and tested for H_2 uptake capability. Bacteroid suspensions from nodules formed by *R. meliloti* strains 102F65 and 102F51 utilized H_2 at rates of 2.4 and 1.0 nmol per h per mg of dry weight, respectively. A continuous amperometric tracing of O_2-dependent H_2 uptake by bacteroids of *R. meliloti* 102F65 is shown in Fig. 2. Since the bacteroids were prepared aerobically, nitrogenase activity was lost, and, as expected, no H_2 evolution from the nitrogenase system was detected. Although the H_2 uptake activity for *R. meliloti* was low, there is no doubt about

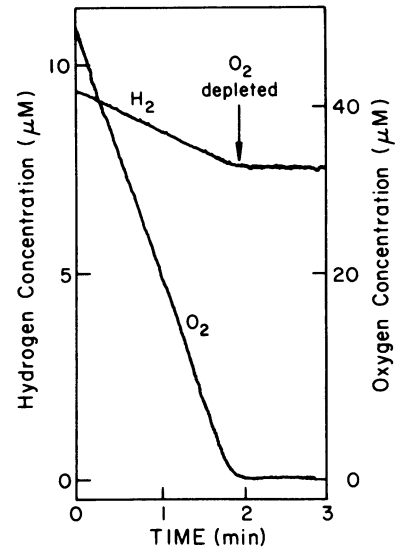

FIG. 2. *Amperometric recordings of H_2 and O_2 uptake rates by bacteroid suspensions from nodules formed by R. meliloti strain 102F65. The reaction was initiated by the addition of 58 ηmol of H_2 and 301 ηmol of O_2 (as saturated solutions) to a final volume of 5.8 ml in a cuvette containing bacteroids equivalent to 150.8 mg (dry weight). The bacteroid suspension was prepared as described in the text.*

the capability of *R. meliloti* bacteroids formed by strain 102F65 or 102F51 to catalyze the oxyhydrogen reaction. The rate of H_2 uptake by bacteroids of *R. meliloti* 102F65 was about 0.04 nmol per min per mg of dry weight. Since *R. japonicum* bacteroids (strain USDA 110) took up H_2 at a rate of 71 nmol per min per mg of dry weight (12), the specific activity in bacteroids of *R. meliloti* 102F65 is about 0.06% of that in bacteroids from soybean nodules formed by *R. japonicum* USDA 110. It is clear that some *R. meliloti* strains possess the capacity to synthesize hydrogenase, but more research is necessary to obtain strains that form bacteroids with sufficient activity to recycle all the H_2 produced by the nitrogenase systems in nodules of alfalfa or clover.

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