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Uptake Hydrogenase Activity Determined by Plasmid pRL6JI in *Rhizobium leguminosarum* Does Not Increase Symbiotic Nitrogen Fixation

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Six mutants of *Rhizobium leguminosarum* 3855 lacking uptake hydrogenase activity (Hup$^-$ phenotype) as a result of Tn*5-mob* mutagenesis of the *hup*-containing plasmid pRL6JI were tested for symbiotic performance on *Pisum sativum* L. and *Vicia benghalensis* L. Three pea cultivars and one vetch line, which induce four different levels of Hup activity in strain 3855, were grown to flowering under microbiologically controlled conditions in the absence of combined N. Direct Kjeldahl N measurements showed that in every case at least one Hup$^-$ mutant fixed as much N_2 as the isogenic Hup$^+$ strain. Measures of C_2H_2 reduction, H_2 evolution, $^3H_2$ incorporation, and plant dry weight were consistent with the interpretation that the oxidation of H_2 produced by the nitrogenase enzyme complex was not necessarily associated with increased N_2 fixation in these symbiotic associations. Tests with a smaller subset of the Hup$^-$ strains under four different root environments ranging from pH 5.0 to 8.2 likewise showed no significant advantage for the isogenic Hup$^+$ strain. It was concluded that the improvements in symbiotic N_2 fixation produced by pRL6JI are associated with some trait other than the Hup$^+$ phenotype.

Rhizobium bacteria within the root nodules of leguminous plants can contain two enzyme systems involved in the metabolism of H_2 (6). The nitrogenase enzyme complex converts N_2 to NH_3 while simultaneously reducing protons to H_2 in all known N_2-fixing systems. Even under 5 MPa of N_2, the system continues to allocate at least 25% of its reductant to H_2 formation (22). In some, but not all, *Rhizobium* bacteria, a separate H_2-uptake (Hup) system oxidizes H_2 to water (11). Three potential advantages of the Hup system in N_2-fixing microorganisms were suggested by Dixon (10): (i) the process utilizes O_2 and thus could protect the O_2-sensitive nitrogenase, (ii) the removal of H_2 could prevent inhibition of nitrogenase by this gas, and (iii) oxidation of H_2 can recover some ATP used by nitrogenase in the formation of H_2. Some of these possible advantages are supported by experimental evidence, but beneficial effects of the Hup system on whole plant growth and on N_2 fixation have been difficult to prove in a rigorous manner (11).

Experiments designed to test the effect of Hup activity on plant growth and on N_2 fixation fall into two categories: (i) comparisons of random collections of Hup$^+$ and Hup$^-$ isolates and (ii) studies with genetically related Hup$^+$ and Hup$^-$ strains. In the case of *Rhizobium japonicum* (1, 13) and mung bean rhizobia (20), collections of Hup$^+$ isolates averaged more N_2 fixation than did Hup$^-$ strains, but analogous comparisons with *R. leguminosarum* showed little symbiotic advantage for Hup$^+$ phenotypes relative to Hup$^-$ strains (17, 23). Recent studies with presumably isogenic mutants of *R. japonicum* supported the earlier work by demonstrating a significant 11% increase in N_2 fixation by a Hup$^+$ revertant of a Hup$^-$ mutant derived originally from the Hup$^+$ strain *R. japonicum* USDA DES122 (12). Work with *R. leguminosarum* showed that the recombinant plasmid pIJ1008 (= pVW5JI, pRL6JI), which confers a Hup$^+$ phenotype, increased N_2 fixation 31 to 128% in already effective, N_2-fixing Hup$^-$ recipient strains (8). Because pIJ1008 carries other symbiotic traits such as nodulation and N_2 fixation (4, 5), it could not be concluded that the improved symbiotic performance resulted only from Hup activity. However, the results did show that no improvement in N_2 fixation was associated with the pVW5JI component; thus, any positive traits presumably were carried on pRL6JI.

The present study was initiated to determine whether the superior symbiotic performance of rhizobial strains containing pRL6JI was associated with the Hup activity conferred by that plasmid. Isogenic Hup$^-$ strains, which had been produced from the Hup$^+$ parent strain 3855 by mutagenesis with Tn*5-mob* (16), were tested for N_2 fixation capacity on pea and vetch plants. If the Hup$^+$ phenotype determined by pRL6JI is responsible for the superior N_2-fixation capacity, then no Hup$^-$ mutant should reduce as much N_2 as the isogenic Hup$^+$ parent. An additional test of selected Hup$^-$ mutants was conducted in various root environments ranging from pH 5.0 to 8.2 to test whether the Hup$^+$ control strains conferred an advantage on plants growing under such conditions.

MATERIALS AND METHODS

Bacterial strains. *R. leguminosarum* strains used are listed in Table 1. The Hup$^-$ mutations were produced by introducing Tn*5-mob* into a streptomycin-resistant isolate of strain 128C53 via the suicide vector pSup5011 (16). The mutations were then transferred to a new genetic background by transduction and selection for kanamycin resistance, determined by Tn*5-mob*. The strains were kindly provided by S. A. Kagan. Physical and genetic evidence suggests that the six Hup$^-$ mutants used in this study are different and that each resulted from a single insertion of Tn*5-mob* into plasmid pRL6JI. All mutations were suppressible by pHU1 (16), a cosmid clone carrying some of the Hup genes from *R. japonicum*.

At the conclusion of each experiment, rhizobia were

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TABLE 1. General characteristics of *R. leguminosarum* strains and their $^3H_2$ incorporation activities in symbioses with the legumes used

Rhizobium strain	Characteristics	H_2 uptake (nmol/mg of nodule [dry wt] per h)			
		JI1205 pea	Alaska pea	Feltham First pea	Vetch
3855	128C53 Str^r	34.6	17.0	10.3	<0.1
518	3855(pRL6JI)::Tn*5-mob*	34.9	16.6	10.2	<0.1
519	3855(pRL13JI)::Tn*5-mob*	31.9	20.6	10.1	<0.1
520	3855(pRL6JI)::Tn*5-mob*	<0.1	<0.1	<0.1	<0.1
521	3855(pRL6JI)::Tn*5-mob*	<0.1	<0.1	<0.1	<0.1
522	3855(pRL6JI)::Tn*5-mob*	<0.1	<0.1	<0.1	<0.1
523	3855(pRL6JI)::Tn*5-mob*	<0.1	<0.1	<0.1	<0.1
524	3855(pRL6JI)::Tn*5-mob*	<0.1	<0.1	<0.1	<0.1
525	3855(pRL6JI)::Tn*5-mob*	<0.1	<0.1	<0.1	<0.1
LSD[a]		2.0	1.0	0.8	<0.1

[a] Least significant difference, $P < 0.05$.

reisolated from root nodules to confirm that the root nodules had been formed by the appropriate strains. Nodules were sent by air from California to Norfolk, United Kingdom, in a desiccated condition suitable for the reisolation of rhizobia. Four nodules from each bacterial strain-plant cultivar treatment were tested. In every case, five clones per nodule were tested for drug resistance markers (15), and one clone per treatment was examined for electrophoretic profiles of plasmids (14).

Plant materials. Seeds of *Pisum sativum* L. cv. JI1205, Alaska, and Feltham First described previously (2) were selected for seed weights of 0.28 to 0.31, 0.20 to 0.23, and 0.32 to 0.35 g, respectively. In addition to the pea cultivars, one pure line of *Vicia benghalensis* L. was also used. The vetch was obtained locally, and a single plant was selected for further seed multiplication. Vetch seeds weighing 0.06 to 0.08 g were used. All seeds were surface sterilized, germinated, planted, and inoculated in sterile Leonard jar assemblies (2). All plants were supplied with sterile N-free nutrient solution (9). Pea plants were grown in 0.75-liter Leonard jars under controlled environmental conditions (2); vetch plants were grown in 1.2-liter Leonard jars from August until November in a greenhouse in which the temperature was maintained between 15 and 25°C. Experiments differed in duration because the plant cultivars developed at different rates. All plants were grown beyond the flowering stage and were harvested the following number of days after germination: Feltham First and Alaska, 38; JI1205, 45; and vetch, 95. Each experiment contained 10 replicate plants inoculated with each bacterial strain and five uninoculated plant controls. Every plant cultivar was tested twice in separate experiments.

To test the relative benefits of Hup$^+$ and Hup$^-$ phenotypes under other environmental conditions, four strains of *R. leguminosarum* (3855, 518, 520, and 525) were inoculated separately onto JI1205 peas with four separate root environments. The experimental procedure was the standard one previously described for JI1205 peas except for changes in the nutrient solution and rooting substrate, which reflected a wide range of possible soil environmental conditions. The four soil and nutrient solution treatments were as follows: (i) vermiculite mixed to contain 10% $CaCO_3$ (wt/wt) and supplied with nutrient solution adjusted to pH 8.1 with 1.0 g of $CaCO_3$ per liter; (ii) vermiculite and nutrient solution adjusted to pH 7.0 with H_2SO_4; (iii) vermiculite and nutrient solution adjusted to pH 5.6 with H_2SO_4; and (iv) baked clay (Saf-T-Sorb; Sierra Chemical Co., West Sacramento, Calif.) ground to a mixture of particle sizes to increase wicking action in the Leonard jars before being adjusted to pH 5.0 with H_2SO_4 and nutrient solution acidified to pH 5.0 with H_2SO_4. Vermiculite was not suitable for treatment iv because it slowly dissolved under the acidic conditions and raised the pH while losing integrity as a rooting medium. No attempt was made to adjust for changes in nutrient availability created by differences in pH among the four treatments.

Physiological analyses. Whole-plant H_2 evolution and C_2H_2-dependent C_2H_4 evolution, less rigorously termed C_2H_2 reduction, were measured sequentially on detached root systems within 23 min after removing shoots at the cotyledonary node (2). Relative efficiency was calculated as $1 - (H_2 \text{ evolved}/C_2H_2 \text{ reduced})$ (21). Hydrogenase activity was measured on separate plants by the following procedure. Root nodules (8 to 10) with approximately 2 mm of root attached were cut from the plant and sealed into 24-ml vials. Then, 10% of the atmosphere in the bottle was exchanged for C_2H_2 to stop H_2 production (2, 3) by nitrogenase. After 5 min, 1.2 ml of a mixture of H_2 and $^3H_2$ with a known specific activity was injected into each vial and allowed to react for 30 min. The nodules were then removed from the vials and refluxed for 10 min in 10 ml of 95% ethanol with an air-cooled condensor. A 2.0-ml sample of the solution was added to 12 ml of Aquasol II (New England Nuclear Corp., Boston, Mass.) and analyzed in a liquid scintillation counter. Data were corrected for background counts, counting efficiency, differential quenching, and surface adsorption phenomena before calculating activity on a per milligram of nodule dry weight per hour basis. Blanks, controls with ^3H_2O, and unnodulated root segments were run in a manner similar to the nodules to provide the necessary correction factors. This procedure proved superior to other methods examined (2, 18) in terms of percent recovery, variability in counts per minute read, and counting efficiencies and ease of analysis.

Plant and data analyses. Plant dry weights were measured after 48 h at 60°C. Reduced N content was determined by the Kjeldahl technique (7). N_2 fixation was calculated by subtracting the original seed N content from total plant N because growth was N_2 dependent. Original seed N values (milligrams) were as follows: JI1205, 8; Alaska, 6; Feltham First, 9; and vetch, 1. Data were examined by one-way analysis of variance to test for significant bacterial strain effects.

RESULTS

In every case, bacterial isolates from root nodules showed the patterns of drug resistance and electrophoretic plasmid profiles expected for the strains inoculated into the Leonard jars. The limited growth and early senescence of uninoculated plants in every experiment also indicated that cross-contamination of Leonard jars had not occurred during experimental manipulations.

The *hup*$^+$ *Rhizobium* genotypes (3855, 518, and 519) showed different levels of Hup activity, as expected, in the three pea cultivars (2) and in the vetch relative to the peas (10) (Table 1). The Hup$^-$ Tn*5-mob* mutants of strain 3855 (strains 520 through 525) exhibited no significant Hup activity in any plant cultivar tested.

The differences in Hup phenotype measured for various *Rhizobium* strains in symbiosis with the JI1205 pea, the plant

cultivar with the highest Hup activity (Table 1), were reflected in highly significant differences in total H_2 evolved from root nodules in that plant cultivar (Table 2). When H_2 evolution was compared in symbiotic associations with similar rates of C_2H_2 reduction and thus presumably with identical rates of H_2 formation by nitrogenase, the results suggested that 90% or more of the H_2 formed by nitrogenase was recovered by the Hup^+ rhizobia in JI1205 root nodules. In other legumes tested, the mean values for strains 3855, 518, and 519 versus strains 520 through 525 of C_2H_2 reduction and H_2 evolution (micromoles per plant per hour) were as follows: Alaska pea, C_2H_2 reduction was 76.3 versus 67.1 and H_2 evolution was 4.9 versus 24.5; Feltham First pea, C_2H_2 reduction was 11.2 versus 8.8 and H_2 evolution was 4.8 versus 6.2; vetch, C_2H_2 reduction was 4.7 versus 4.9 and H_2 evolution was 3.5 versus 4.1.

There was no evidence suggesting that the Hup^+ rhizobia were symbiotically superior to Hup^- bacteria in any of the four host legumes tested during eight experiments involving 760 plants (Tables 3 and 4). In every case, at least one Hup^- mutant fixed as much N_2 (Table 3) and produced a plant with as much dry matter (Table 4) as did strain 518, the most appropriate control. In fact, when the results were averaged across all Tn*5-mob*-induced Hup^- mutants, the mean values were numerically larger than the data from plants nodulated by strain 518. The symbiotic performance of the average Hup^- strain also exceeded that of the parent strain 3855 in all cases except with the Feltham First cultivar. There was no significant difference in shoot/root ratios between plants nodulated with Hup^+ and Hup^- rhizobia.

Tests with various root environments defined by pH and substrate differences showed no significant decrease in plant dry weight or in N_2 fixed for JI1205 peas nodulated by Hup^- strains relative to Hup^+ controls.

DISCUSSION

Data from these experiments (Tables 3 and 4) allow us to conclude that determinants for the Hup system carried on pRL6JI clearly do not contribute to improvements in symbiotic performance associated with this plasmid (8). A positive role in N_2 fixation for the Hup^+ phenotype determined by pRL6JI would be indicated only if Hup^+ strain 518 had fixed more N_2 than every Hup^- mutant tested. The fact that at least one Hup^- strain fixed as much or more N_2 than did strain 518 in every legume examined is critical proof that the elimination of Hup activity is not necessarily associated with a decline in N_2 fixation. The fact that the mean value for N_2 fixation by all Hup^- strains exceeded the amount of N_2 fixed by strain 518 in every legume supports the overall conclusion but is not a necessary condition for that proof. Likewise, a comparison between the amount of N_2 fixed by Hup^- strains and the amount fixed by the parent strain 3855 is not a rigorous test of the hypothesis but does support the conclusion that Hup activity is not a positive component of N_2 fixation in that strain.

There is no evidence to suggest that Tn*5-mob* itself contributed to the symbiotic performance of *R. leguminosarum* and thus counterbalanced any negative effects associated with the Hup^- phenotype. The Tn*5-mob* element present in pRL6JI (strain 518) or pRL13JI (strain 519) had no significant effect on Hup activity (Table 1), H_2 evolution or relative efficiency (Table 2), total N_2 fixed (Table 3), or plant dry weight (Table 4) relative to the parent strain 3855. The marginally significant increase in C_2H_2-reduction activity of strain 518 relative to strain 3855 in the JI1205 pea (Table 2)

TABLE 3. N_2 fixed by experimental strains of *R. leguminosarum* in symbioses with various legumes

Rhizobium strain	N_2 (mg) fixed			
	JI1205 pea	Alaska pea	Feltham First pea	Vetch
3855	93	62	98	276
518	82	62	86	255
519	100	61	91	261
520	97	66	99	315
521	108	65	94	268
522	96	68	92	276
523	96	75	75	380
524	99	62	97	319
525	101	67	92	284
LSD[a]	11	8	14	70

[a] Least significant difference, $P < 0.05$.

TABLE 2. Root nodule activities of experimental *R. leguminosarum* strains in symbiosis with the JI1205 pea, the legume that produced the greatest Hup activity in Hup^+ bacterial strains

Rhizobium strain	Hup phenotype	Root nodule activity (μmol/plant per h)		Relative efficiency
		C_2H_2 reduction	H_2 evolution	
3855	+	63.5	3.1	0.95
518	+	88.9	6.0	0.91
519	+	76.4	5.7	0.93
520	−	76.3	22.6	0.70
521	−	73.3	24.7	0.66
522	−	57.9	24.9	0.55
523	−	60.6	25.9	0.56
524	−	69.9	27.4	0.59
525	−	54.7	22.0	0.55
LSD[a]		25.0	8.4	0.14

[a] Least significant difference, $P < 0.05$.

TABLE 4. Total dry weight of N_2-dependent legumes grown in symbiosis with experimental strains of *R. leguminosarum*

Rhizobium strain	Dry wt (g)			
	JI1205 pea	Alaska pea	Feltham First pea	Vetch
3855	2.92	2.10	2.83	7.01
518	2.79	2.19	2.50	6.54
519	3.00	2.12	2.63	6.74
520	2.94	2.18	2.86	8.10
521	3.32	2.17	2.69	6.86
522	2.98	2.25	2.71	6.88
523	2.96	2.48	2.18	8.78
524	3.00	2.14	2.67	8.00
525	3.24	2.25	2.67	7.12
LSD[a]	0.39	0.25	0.42	1.27

[a] Least significant difference, $P < 0.05$.

was not reflected in the total N_2 fixed (Table 3) and was not measured in a second experiment.

Another test for the importance of Hup activity in *R. leguminosarum* was imposed by comparing the effects of bacterial strains on different legume hosts. If Hup activity contributes significantly to symbiotic performance in strains containing plasmid pRL6JI, then the effect should be most prominent in the JI1205 pea, in which Hup activity is greatest, and should diminish proportionately in the Alaska pea, the Feltham First pea, and the vetch, which had approximately 50, 30, and 0%, respectively, of the Hup activity seen in the JI1205 pea (Table 1). No such trend was apparent in the N_2 fixation values (Table 3).

One might suggest that the Hup^+ phenotype confers an advantage on symbiotic associations that would be apparent only under less ideal conditions, such as low or high pH. However, tests with bacterial strains 3855, 518, 520, and 525 showed that in every pH-rooting substrate treatment at least one Hup^- strain fixed as much, or not significantly less, N_2 than control strain 518. Obviously, these treatments did not test all possible rooting environments that might affect the symbiotic performance of a *Rhizobium* strain, but they did provide a wide range of conditions over which the Hup^- strains were not at a clear disadvantage in the symbiotic mode. That fact, along with the observation that pea cultivars can alter the Hup activity of *hup*$^+$ *Rhizobium* genotypes without increasing growth and N_2 fixation relative to plants of the same cultivar nodulated by isogenic Hup^- rhizobia (Table 1), suggests that Hup activity confers few advantages on symbiotic *R. leguminosarum*. Perhaps more attention should be given to the possibility that Hup activity benefits rhizobia growing heterotrophically in the soil.

Although we can conclude from this study that the Hup activity determined by pRL6JI is not a critical component of the symbiotic traits borne on that plasmid, it is still possible that Hup systems in other strains of *R. leguminosarum* may be associated with increased N_2 fixation. This qualification of the present results is necessary because it has been reported that H_2 oxidation in *R. leguminosarum* 128C53 is not strongly coupled to ATP synthesis (19). Other potential benefits of Hup systems, such as decreasing concentrations of H_2 and O_2 within *Rhizobium* cells, presumably still functioned within strain 3855 and therefore were tested in this study. Likewise, it is not possible to extend these results to other species of *Rhizobium*, different host legumes, or other environmental conditions.

One striking conclusion from the present study must not be overlooked. All data indicate that Hup activity conferred by pRL6JI does not increase N_2 fixation, yet previous experiments indicated that genetic determinants on pRL6JI promoted N_2 fixation markedly (8). Therefore, pRL6JI must contain genes for one or more unknown, but very beneficial, symbiotic traits.

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LITERATURE CITED

1. **Albrecht, S. L., R. J. Maier, F. J. Hanus, S. A. Russell, D. W. Emerich, and H. J. Evans.** 1979. Hydrogenase in *Rhizobium japonicum* increases nitrogen fixation by nodulated soybeans. Science **203**:1255–1257.
2. **Bedmar, E. J., S. A. Edie, and D. A. Phillips.** 1983. Host plant cultivar effects on hydrogen evolution by *Rhizobium leguminosarum*. Plant Physiol. **72**:1011–1015.
3. **Bethlenfalvay, G. J., and D. A. Phillips.** 1979. Variation in nitrogenase and hydrogenase activity of Alaska pea root nodules. Plant Physiol. **63**:816–820.
4. **Brewin, N. J., N. J. Dibb, and G. Hombrecher.** 1983. Natural variation in *Rhizobium* plasmids, p. 113–120. *In* A. Puhler (ed.), Molecular genetics of the bacteria plant interaction. Springer-Verlag KG, Berlin.
5. **Brewin, N. J., E. A. Wood, A. W. B. Johnston, N. J. Dibb, and G. Hombrecher.** 1982. Recombinant nodulation plasmids in *Rhizobium leguminosarum*. J. Gen. Microbiol. **128**:1817–1827.
6. **Burns, R. C., and R. W. F. Hardy.** 1975. Nitrogen fixation in bacteria and higher plants. Springer-Verlag, New York.
7. **Burris, R. H., and P. W. Wilson.** 1957. Methods for measurement of nitrogen fixation. Methods Enzymol. **4**:355–366.
8. **DeJong, T. M., N. J. Brewin, A. W. B. Johnston, and D. A. Phillips.** 1982. Improvement of symbiotic properties in *Rhizobium leguminosarum* by plasmid transfer. J. Gen. Microbiol. **128**:1829–1838.
9. **DeJong, T. M., and D. A. Phillips.** 1981. Nitrogen stress and apparent photosynthesis in symbiotically grown *Pisum sativum* L. Plant Physiol. **68**:309–313.
10. **Dixon, R. O. D.** 1972. Hydrogenase in legume root nodule bacteroids: occurrence and properties. Arch. Mikrobiol. **85**:193–201.
11. **Eisbrenner, G., and H. J. Evans.** 1983. Aspects of hydrogen metabolism in nitrogen-fixing legumes and other plant-microbe associations. Annu. Rev. Plant Physiol. **34**:105–136.
12. **Evans, H. J., F. J. Hanus, S. A. Russell, A. R. Harker, G. R. Lambert, and D. A. Dalton.** 1985. Biochemical characterization, evaluation, and genetics of H_2 recycling in *Rhizobium*, p. 3–11. *In* P. W. Ludden and J. E. Burris (ed.), Nitrogen fixation and CO_2 metabolism. Elsevier Science Publishing Co., New York.
13. **Hanus, F. J., S. L. Albrecht, R. M. Zablotowicz, D. W. Emerich, S. A. Russell, and H. J. Evans.** 1981. Yield and N content of soybean seed as influenced by *Rhizobium japonicum* inoculants possessing the hydrogenase characteristic. Agron. J. **73**:368–372.
14. **Hirsch, P. R., A. W. B. Johnston, N. J. Brewin, M. Van Montagu, and J. Schell.** 1980. Physical identification of bacteriocinogenic, nodulation, and other plasmids in strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. **120**:403–412.
15. **Josey, D. P., J. L. Beynon, A. W. B. Johnston, and J. E. Beringer.** 1979. Strain identification in *Rhizobium* using intrinsic antibiotic resistance. J. Appl. Bacteriol. **46**:343–350.
16. **Kagan, S. A., and N. J. Brewin.** 1985. Mutagenesis of a *Rhizobium* plasmid carrying hydrogenase determinants. J. Gen. Microbiol. **131**:1141–1147.
17. **Nelson, L. M.** 1983. Hydrogen recycling by *Rhizobium leguminosarum* isolates and growth and nitrogen contents of pea plants (*Pisum sativum* L.). Appl. Environ. Microbiol. **45**:856–861.
18. **Nelson, L. M., and J. J. Child.** 1981. Nitrogen fixation and hydrogen metabolism by *Rhizobium leguminosarum* isolates in pea root nodules. Can. J. Microbiol. **27**:1028–1034.
19. **Nelson, L. M., and S. O. Salminen.** 1982. Uptake hydrogenase activity and ATP formation in *Rhizobium leguminosarum* bacteroids. J. Bacteriol. **151**:989–995.
20. **Pahwa, K., and R. C. Dogra.** 1981. H_2-recycling system in mungbean *Rhizobium* in relation to N_2-fixation. Arch. Microbiol. **129**:380–383.
21. **Schubert, K. R., and H. J. Evans.** 1976. Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc. Natl. Acad. Sci. USA **73**:1207–1211.
22. **Simpson, F. B., and R. H. Burris.** 1984. A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. Science **224**:1095–1097.
23. **Truelsen, T. A., and R. Wyndaele.** 1984. Recycling efficiency in hydrogenase uptake positive strains of *Rhizobium leguminosarum*. Physiol. Plant. **62**:45–50.