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Revertible Hydrogen Uptake-Deficient Mutants of *Rhizobium japonicum*†

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We have developed mutants of *Rhizobium japonicum* which are deficient in H_2 uptake capacity (Hup^-) and which spontaneously revert to the parent type at a frequency consistent with that of a single-point mutation (ca. 1.0×10^{-9}). The mutagenesis by nitrous acid and the selection of the Hup^- phenotype by using penicillin and chemolithotrophy as enrichment for chemolithotrophy-deficient strains are described. Two mutants retain low but reproducible levels of ribulose bisphosphate-dependent CO_2 fixation when grown on a low-carbon medium under an atmosphere of 1% O_2, 4% H_2, 5% CO_2, and 90% N_2. Neither O_2 nor the artificial electron acceptors phenazine methosulfate or methylene blue supported detectable H_2 uptake by the free-living Hup^- mutants or by their bacteroids. Plant growth experiments under bacteriologically controlled conditions were conducted to assess the mutants' performance as inocula for soybean plants. Plants inoculated with Hup^- strains had lower dry weights and contained less total N than did plants inoculated with the parent Hup^+ strain. Use of either the Hup^- mutants or the Hup^+ parent strain as inocula, however, did not significantly affect the acetylene-reducing activity or the fresh weight of nodules. These results, obtained with apparently isogenic lines of H_2 uptake-deficient *R. japonicum*, provide strong support for a beneficial role of the H_2 uptake phenotype in legume symbiosis.

Nitrogenase catalyzes an ATP-dependent reduction of protons and a consequent evolution of H_2 during N_2 fixation, resulting in an inefficient use of energy (9, 29). Some strains of *Rhizobium* sp. form nodules in which the H_2 produced from the nitrogenase reaction is oxidized via an uptake hydrogenase, thus preventing H_2 loss (5). H_2 recycling in these Hup^+ strains has several effects that are beneficial to efficient symbiosis: (i) ATP generated during H_2 utilization may be used in N_2 fixation; (ii) additional protection from O_2 inactivation is provided for the labile nitrogenase; and (iii) carbon substrates are conserved (7, 8, 28).

Hydrogenase activity is expressed in *Rhizobium japonicum* nodule bacteroids and in free-living cultures under defined conditions (18, 21, 24). Several factors, including induction by H_2 and repression by O_2 and carbon substrates, regulate the formation of the H_2 uptake system in free-living *R. japonicum* (19, 21, 22, 27). Moreover, Hup^+ *R. japonicum* strains grow chemolithotrophically, using H_2 as an energy source (14) and fixing CO_2 via RuBP carboxylase (17).

Greenhouse experiments which compared wild-type *R. japonicum* strains having an H_2 uptake capacity (Hup^+) with strains lacking this ability (Hup^-) as inocula for soybeans support the contention, but do not prove, that benefits of H_2 uptake may be realized (1, 30).

Maier et al. (23) described mutants of *R. japonicum* that lack the H_2 uptake capability in both free-living and bacteroid forms. Soybean plants inoculated with these mutants fixed less N_2 than did plants inoculated with the Hup^+ parent, but repeated attempts to obtain revertants of these mutants failed, and thus their isogenicity with their Hup^+ parent is doubtful.

We now report the mutagenesis of free-living cultures of Hup^+ *R. japonicum* by the use of nitrous acid to obtain Hup^- mutants. These mutants, which were obtained by the procedure to be described, revert to the parent phenotype and therefore should be useful in the assessment of the benefits of H_2 recycling to N_2 fixation by legumes. Three such mutant strains have been partially characterized.

MATERIALS AND METHODS

Chemicals. $NaH^{14}CO_3$ (specific activity, 48 mCi/mmol) was obtained from New England Corp., Boston, Mass. C_2H_4 was obtained from Matheson Gas Products, East Rutherford, N.J., and other gases were

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purchased from Airco Industrial Gases Division, Vancouver, Wash. C_2H_2 was generated from calcium carbide (4). *N*-methylphenazinium methosulfate (phenazine methosulfate; PMS), penicillin G, kanamycin sulfate, streptomycin sulfate, lysozyme, bovine serum albumin, and methylene blue (MB) were obtained from Sigma Chemical Co., St. Louis, Mo. Nitrous acid was prepared from sodium nitrite in 0.1 M acetate, pH 4.7, as described by Miller (26).

Strains, media, and culture conditions. *R. japonicum* SR, a spontaneous streptomycin (250 μg/ml)- and kanamycin (100 μg/ml)-resistant mutant of *R. japonicum* USDA DES 122, was used throughout this work (23). Growth conditions were as described by Lepo et al. (17). Derepression of hydrogenase in free-living cultures was effected by use of the H_2 uptake medium (HUM) as described by Maier et al. (21). Stock cultures were also maintained and plate counts were done on HUM medium. A medium containing arabinose, mineral salts, and 0.1 g of yeast extract per liter, referred to as AIEHM (6), was used for determinations of growth rates of mutants.

Rates of H_2 uptake and evolution. H_2 uptake rates were measured amperometrically as described previously (13), using saturating concentrations of H_2 and O_2. H_2 evolution from nodules was measured amperometrically in air as described by Schubert and Evans (29).

RuBP carboxylase assay. The activity of RuBP carboxylase in extracts was determined by the method of McFadden et al. (25), and in cell suspensions the activity was determined by the method of Tabita et al. (34).

Protein determinations. Suspensions of free-living cells and bacteroids were digested by the method of Stickland (33). Protein in free-living cells was determined by the method of Lowry et al. (20), and in bacteroid suspensions it was determined by the microbiuret method of Goa (12). Bovine serum albumin was used as a standard.

Characterization of Hup^- mutants as inocula for soybeans. Surface-disinfected soybean seeds (35) were germinated (48 h) on plates of mineral salts-agar and then soaked for 20 min in 3-day-old HUM broth cultures of parent SR and Hup^- strains of *R. japonicum*. Inoculated soybean [*Glycine max* (L.) Merr. var. Wilkin] seedlings were grown in modified Leonard jars under axenic conditions (35) for 28 days in growth cabinets as previously described (1). Acetylene-reduction activities of excised nodules were determined as described previously by Schwinghamer et al. (31). Nodules were harvested, and bacteroids were prepared aerobically by the procedures of Evans et al. (10) with the modifications of Ruiz-Argüeso et al. (27). H_2 uptake by bacteroids with alternative receptors were carried out as outlined by Ruiz-Argüeso et al. (27). Plant tissues were dried to constant weight at 80°C. Nitrogen in dry plant materials was determined with an Orion ammonia electrode after Kjeldahl digestion of 200-mg samples (3).

Mutagenesis of *R. japonicum* SR. Nitrous acid mutagenesis was carried out by the method of Kaudewitz (15) as outlined by Miller (26), with the exception that a 30-min exposure to the mutagen was used. Chemolithotrophically grown cultures (50 ml) of *R. japonicum* SR in the late-logarithmic phase were harvested in sterile centrifuge tubes (25 ml of culture per tube). Each pellet was suspended in 10 ml of 0.1 M acetate buffer (pH 4.6), centrifuged, and suspended in 2 ml of freshly prepared nitrous acid (50 mM in 0.1 M acetate, pH 4.6). A sample for plate counts was taken immediately and placed in a chemolithotrophic medium blank. Exposure to nitrous acid was continued for 30 min at 30°C on a gyratory shaker. Exposure to the mutagen was terminated by the addition of 10 ml of the chemolithotrophic medium (pH 7.0). The viability of the cultures was found to be typically reduced by a factor of 10^4 to 10^5.

Selection by Hup^- mutants. Dilutions of mutagenized cells of 10^{-2}, 10^{-4}, and 10^{-6} were made into 50-ml volumes of chemolithotrophic medium containing 300 μg of penicillin G per ml. These cultures were incubated under chemolithotrophic conditions as described previously (17) for 6 to 8 days; then they were vacuum filtered onto membrane pads (5 cm; 0.22-μm pore size). At least 250 ml of sterile chemolithotrophic medium was used to wash the membrane-collected cells, thus removing the penicillin. Each pad was incubated on the surface of a HUM agar plate in air at 30°C. Colonies on these plates were replica plated on chemolithotrophic medium with and without 0.5% glucose. The plates were incubated under an atmosphere permitting chemolithotrophic growth (17). Colonies formed on chemolithotrophic medium with, but not without, glucose were selected as putative Hup^- strains. Clones which were chemolithotrophy negative and lacked H_2 uptake capacity, as determined amperometrically, were selected for further study.

Two changes in the above protocol, which enhanced the efficiency of the method, were incorporated into later selection procedures. (i) After mutagenesis, cultures were grown chemolithotrophically for 4 days in the absence of penicillin to allow for segregation of the mutant genome and for depletion of the chemolithotrophic capacity. (ii) The addition of 100 μg of lysozyme per ml with the penicillin improved the killing efficiency of the antibiotic (16).

Reversion rates. The rate of reversion to the parental phenotype was estimated after growth of the Hup^- mutant cells in 9 ml of HUM broth cultures for 3 days at 28°C. The cells were washed twice in sterile 50 mM potassium phosphate (pH 6.05) containing 0.85% NaCl to remove carbon substrates. Suspensions of these cells in phosphate-buffered saline served as inocula for plates of chemolithotrophic medium. Viable cell counts of the inoculum were determined by plating on HUM medium. Ninety-five plates inoculated with each mutant strain (at 10^8 cells per plate) and two plates inoculated with Hup^+ strain SR as a positive control were incubated in a polyethylene bucket with a volume of about 20 liters. Thus, approximately 10^{10} cells of each mutant were examined for reversion to chemolithotrophy. The gas phase in each bucket was maintained at 1% O_2, 4% H_2, 5% CO_2, and 90% N_2. After 16 days of incubation at 28°C the buckets were opened, and colonies which appeared within the "lawn" were counted and selected for characterization as potential revertants. The periphery of the area where the inoculum was spread produced several pseudorevertant colonies that showed no mea-

surable ability to oxidize H_2. Colonies of this type apparently survived by scavenging organic carbon from impurities in the medium.

RESULTS

Derivation and growth characteristics of mutants. Colonies of *R. japonicum* which survived the penicillin selection procedure under chemolithotrophic conditions (see above) and failed to grow on plates of chemolithotrophic medium were streaked on HUM plates for further study. These chemolithotrophy-negative cells arose at a frequency of 10^{-5} among the survivors of the nitrous acid treatment. Isolated colonies of the chemolithotrophy-negative mutants were tested for resistance to kanamycin and streptomycin. After 3 days of growth on HUM broth at 28°C, cultures were streaked onto HUM plates with 100 μg of kanamycin and 250 μg of streptomycin per milliliter. Resistance to both antibiotics was shown in all of the mutant strains described in this study. One colony type was observed in streaks of the mutants, and the colony morphology was indistinguishable from that of the wild-type *R. japonicum* SR.

HUM agar plates also were spread with 0.1 ml of a suspension of 3-day-old HUM broth cultures of the chemolithotrophy-negative mutants and were incubated for 5 days at 28°C in gas-tight jars under an atmosphere of 1% O_2, 5% H_2, 89% N_2. Suspensions of cells made from growth on these plates were derepressed and assayed for hydrogenase. Some of the chemolithotrophy-negative clones possessed hydrogenase activities comparable to those of the wild-type strain. These clones may have been deficient in enzymes unique to the carbon fixation mechanism and were put aside for future study.

Three Hup^- strains (designated PJ17, PJ18, and PJ20) which were products of separate replicate selections were selected for characterizations. Since we wished to obtain hydrogenase mutants that were otherwise isogenic with the parent strain to study the potential benefits of the uptake-hydrogenase system, it was imperative that reversion rates of the mutants to the parental phenotype be established.

Reversion rates ranged from 9×10^{-10} to 1.3×10^{-9} (Table 1). Since only those colonies that were obtained in the central lawn area were examined as potential revertants and since it is likely that some of the colonies around the periphery were indeed revertants, these rates are considered minimal. In a separate experiment with mutants PJ17 and PJ18, revertants to the Hup^+ parent phenotype occurred at a rate of approximately 1×10^{-9}. All of the Hup^- mutant strains and the revertants derived from them

TABLE 1. *Growth and reversion rates of Hup^- mutants of R. japonicum compared with the parent strain SR*

Strain	Generation time (h)[a]		Reversion rate
	AIEHM	HUM	
SR	9.0	7.8	
PJ17	9.2	8.0	1.0×10^{-9}
PJ18	8.3	7.0	1.3×10^{-9}
PJ20	12.5	10.0	9.0×10^{-10}

[a] Cells from mid-logarithmic phase of growth in HUM broth were inoculated into HUM and AIEHM broth media (1% inoculum), and optical densities at 540 nm were measured periodically until stationary phase was attained. Generation times were calculated from growth curves under these conditions.

retained both antibiotic resistances and displayed colony morphology indistinguishable from that of the SR parent strain. Furthermore, the RuBP carboxylase and hydrogenase activities of revertants from mutants PJ17 and PJ18 were (within experimental error) the same as those of the parent SR strain. Also shown in Table 1 are generation times for the parent and mutant strains, calculated from their growth in HUM and AIEHM media. Rates similar to or higher than those obtained with the parent SR strain were found for mutants PJ17 and PJ18, whereas PJ20 grew noticeably more slowly than the parent strain in these media.

Hydrogenase and RuBP carboxylase activities of cultured cells. H_2 uptake activities of the mutants and the parent SR strain were compared in assays utilizing O_2, PMS, or MB as electron acceptors. These acceptors were used because Ruiz-Argüeso et al. (27) showed that both PMS and MB functioned in place of O_2 as electron acceptors for the hydrogenase in *R. japonicum* bacteroids. Furthermore, other Hup^- mutants of *R. japonicum* which failed to oxidize H_2 with O_2 as the acceptor, but catalyzed PMS-dependent H_2 oxidation, have been isolated in our laboratory. Since none of the mutants showed PMS-, MB-, or O_2-dependent H_2 oxidation, we conclude that the H_2 activation step of the H_2 oxidation systems is not functional.

Since previous investigations by Simpson et al. (32) have shown coordinate induction of hydrogenase and RuBP carboxylase in a Hup^+ strain of *R. japonicum,* it was of interest to determine whether RuBP carboxylase activity could be expressed in the Hup^- mutant strains. RuBP carboxylase specific activities of toluene-treated cell suspensions of Hup^- mutants and the parent SR strain are presented in Table 2. Easily measured activity was observed in the parent SR strain, and low but reproducible ri-

bulose bisphosphate-dependent activity (400- to 500-fold the minus-RuBP control) was detected in mutants PJ17 and PJ20. Activity in the suspensions of PJ18 was below the detectable limit of the method; however, in a separate experiment, low activity was detected in mutant PJ18.

Since the Hup$^-$ mutants are incapable of chemolithotrophic growth, conditions for derepression of RuBP carboxylase must necessarily be compromised. The derepression regime (21) limits the exposure to exogenous carbon while exposing the cells to inducing conditions which include, in addition to a limited organic carbon supply, a source of H_2 and limited O_2.

TABLE 2. *Uptake hydrogenase and RuBP carboxylase activities in free-living cells of Hup$^-$ mutants and the Hup$^+$ parent strain of R. japonicum*

Strain	H_2 uptake (nmol/min per mg of protein) with[a]:			RuBP carboxylase[b] (nmol of CO_2 fixed/min per mg of protein)
	O_2	PMS	MB	
SR	74.5	82.7	97.2	14.2
PJ17	<0.5	<0.5	<0.5	1.7
PJ18	<0.5	<0.5	<0.5	<0.5
PJ20	<0.5	<0.5	<0.5	1.6

[a] Cells were plated on HUM medium and incubated for 5 days under derepressing conditions at 28°C. Suspensions of cells from plates were made in potassium phosphate-$MgCl_2$ buffer, and hydrogenase was assayed with a saturating concentration of O_2 or with 1.0 mM PMS or MB in the absence of O_2.

[b] Cells from two HUM plates of each strain, derepressed as described in footnote *a*, were washed twice and then suspended in 20 ml of sterile potassium phosphate-$MgCl_2$ buffer. Each suspension was incubated for 36 h at 25°C in sterile 70-ml bottles under an atmosphere of 1% O_2, 4% H_2, 5% CO_2, and 90% N_2. Cells were washed once in 20 mM Tris-sulfate (pH 8.0) containing 1 mM EDTA, 10 mM $MgCl_2$, and 50 mM $NaHCO_3$. The pellet suspended in 0.5 ml of this solution was toluene treated and assayed for RuBP carboxylase.

The addition of 10 mM succinate to suspensions of derepressed mutant cells just before RuBP carboxylase assays failed to stimulate RuBP carboxylase activities.

Behavior of mutants under symbiotic conditions. Several characteristics of the mutants and parent SR strain as inocula for symbiotically cultured soybean plants are compared in Table 3. Nodules formed by the Hup$^+$ SR strain evolved no measurable H_2, whereas nodules formed by the Hup$^-$ mutants PJ17 and PJ18 lost H_2 at substantial rates. Assuming that the C_2H_2 reduction rate is a measure of the total electron flux through nitrogenase (11, 29), these H_2 losses amount to 55 and 44% of the nitrogenase electron flux for nodule bacteroids formed by mutants PJ17 and PJ18, respectively. Although the C_2H_2 reduction rates of nodules formed by mutants and the parent SR strain showed apparent differences, the variation among samples from replicate cultures was such that the differences were not statistically significant.

Bacteroid preparations from nodules formed by PJ17, PJ18, and the parent strain SR were prepared aerobically, and H_2 uptake capacity was assayed with O_2, PMS, and MB as electron acceptors. Suspensions of bacteroids formed by this SR strain were diluted 50-fold (to about 100 μg of protein per ml) to obtain measurable rates, whereas bacteroids formed by mutant strains were assayed without dilution (3 to 4 mg of protein per ml). None of the bacteroids formed by mutant strains showed detectable H_2 uptake activity with any of the three acceptors. In contrast, bacteroids formed by the parent SR strain showed high H_2 uptake rates with all three acceptors. These results are consistent with the reported nitrogenase-dependent H_2 losses from nodules formed by the different strains (Table 3). A second experiment (not reported in detail), in which the SR parent strain and mutants PJ17, PJ18, and PJ20 were compared as inocula for

TABLE 3. *Comparison of properties of nodules and bacteroids formed by Hup$^-$ mutants and parent strain SR of R. japonicum*[a]

Strain	Measurements on intact nodules (nmol/h per g [fresh wt])		Bacteroid H_2 uptake (nmol/min per mg of protein) with:		
	H_2 evolution	C_2H_2 reduction	O_2	PMS	MB
SR	<0.5	16.1 ± 0.8	43.3 ± 2.6	45.0	46.0
PJ17	8.0 ± 1.5	14.5 ± 0.8	<0.5	<0.5	<0.5
PJ18	5.2 ± 1.4	11.9 ± 1.4	<0.5	<0.5	<0.5

[a] H_2 evolution and C_2H_2 reduction of 1-g samples of nodules in 21-ml vials and rates of H_2 uptake by bacteroid suspensions determined at 25°C are shown. H_2 uptake by bacteroids was determined as described in Table 2, footnote *a*. Data are means of determinations on ten replicate cultures (± standard error of the mean). Differences in rates of C_2H_2 reduction by the three treatments and differences in rates of H_2 evolution from nodules formed by mutants PJ17 and PJ18 are not significant at the 0.05 level.

soybeans, produced results that were consistent with those presented in Table 3.

The effects of mutants PJ17, PJ18, and the parent SR strain as inocula for soybeans on growth and N content of plants are presented in Table 4. With the Hup$^+$ parent strain SR as the inoculum, plants with higher dry weights and total N contents than those inoculated with either of the Hup$^-$ mutant strains were produced. The total N fixed by plants inoculated with Hup$^+$ parent strain SR was significantly higher ($P \leq 0.05$) than the N contents of plants inoculated with either Hup$^-$ mutant strain. The dry weights of plants inoculated with the parent strain SR were significantly higher ($P \leq 0.05$) than the weights of plants inoculated with the Hup$^-$ mutant PJ18. The effect of treatment on the fresh weight of nodules was not significant.

DISCUSSION

The availability of Hup$^-$ mutants isogenic with the Hup$^+$ parent is necessary to convincingly demonstrate the benefits of H_2 recycling to the nitrogen fixation system in nodulated legumes. Mutants which do not revert may have multiple lesions or deletions of large amounts of genetic information. The effects of hydrogenase deficiency in such mutants may thus be obscured during the symbiotic relationship by other physiological defects. Since the development of methods for genetic exchange among *R. japonicum* strains seems imminent, mutants will be useful for the investigation of the regulation of the H_2 uptake system. Moreover, the methods for selection of mutants described here should be applicable to the selection of auxotrophic mutants.

The Hup$^-$ strains described in this paper retain the two antibiotic markers and, to our knowledge, the other characteristics of the parental phenotype except the hydrogenase system. Mutants PJ17 and PJ18 grow at rates comparable to that of the parent strain; however, for reasons not apparent PJ20 grew somewhat more slowly than did parent strain SR in the two media and thus was not utilized in the evaluation of the strains under symbiotic conditions.

TABLE 4. *Effect of Hup$^-$ mutants and the parent strain SR on growth parameters and nitrogen fixation by soybeans*[a]

Inoculating strain	Total dry wt[b] (g)	Fresh wt of nodules (g)	Total N[b] (mg)
SR (Hup$^+$)	4.45 ± 0.19	1.17 ± 0.07	88.5 ± 4.51
PJ17 (Hup$^-$)	4.17 ± 0.16	1.33 ± 0.05	70.8 ± 4.56
PJ18 (Hup$^-$)	3.46 ± 0.19	1.33 ± 0.03	58.6 ± 7.29
None	2.45 ± 0.13	0	11.8 ± 1.39

[a] Soybean seeds (cv Wilkin) were inoculated and plants were grown as described. Values, except for uninoculated controls, are means of ten replicate Leonard cultures each with two plants per jar. Data for the uninoculated controls are means of five replicate cultures (± standard error of the mean). These are not included in the statistical analyses.

[b] The mean dry weights of plants inoculated with strain SR were different from that of plants inoculated with PJ18 at the 0.05 level. Differences between yields of plants inoculated with SR and PJ17 were not significant; however, total N fixed by plants inoculated with either strain PJ17 or PJ18 were significantly less ($P \leq 0.05$) than that of plants inoculated with strain SR.

None of the three mutants characterized here showed H_2 uptake activity after derepression under conditions that promote the expression of high activity in Hup$^+$ strains. The addition of PMS or MB as artificial electron acceptors to suspensions of either bacteroids or hydrogenase-induced free-living Hup$^-$ mutants resulted in no hydrogenase activity. Since artificial acceptors, such as PMS and MB, accept electrons from hydrogenase at a point that bypasses most or all of the electron transport components between hydrogenase and O_2 (27) and since purified hydrogenase from *R. japonicum* bacteroids transfers electrons directly to acceptors such as PMS and MB (2), our data indicate that the Hup$^-$ mutants are deficient in the hydrogenase activating system per se.

If these mutants are indeed the result of single-point defects, they should retain the genetic capacity to synthesize RuBP carboxylase unless the lesion exists in a regulatory gene or site common to both the H_2 uptake system and RuBP carboxylase. Reversion to the Hup$^+$ phenotype should then restore chemolithotrophic ability as well. Cells surviving penicillin counterselection, however, could be defective in either the energy-generating or the CO_2-fixing components of the Calvin cycle. The work of Simpson et al. (32) has suggested that coordinate repression and induction of RuBP carboxylase and hydrogenase occurs in a Hup$^+$ 122 DES strain of *R. japonicum* with the addition of succinate. The Hup$^-$ mutants of *R. japonicum* used by Simpson (32) failed to revert to the Hup$^+$ phenotype and did not express RuBP carboxylase CO_2-fixing activity (32). An adaptation of the methods of Simpson et al. (32) has enabled us to show low but reproducible activity of RuBP carboxylase in PJ17 and PJ20. In a separate experiment (not reported), low activity of RuBP carboxylase was detected in mutant PJ18 as well. It seems probable that the low activities of RuBP carboxylase in the Hup$^-$ mutants PJ17 and PJ18 are associated with the regulatory system. For example, hydrogenase and RuBP carboxylase are not expressed unless H_2 is supplied, O_2 is limited, and a very low level of organic carbon is provided (32). Since chemolithotrophy-negative mutants require carbon substrates for growth, RuBP carboxylase activ-

ity might not be detected in Hup^- mutants unless the level of carbon substrates approached a narrow critical optimum.

Of the colonies which showed apparent chemolithotrophic growth (about 70 in 10^{10} cells plated onto chemolithotrophic medium), only 1 in 7 showed hydrogenase activity. Thus, reversion rates in the order of 1 in 10^9 cells were observed. These rates are perhaps lower than would be expected, but the restoration of chemolithotrophic capacity and normal levels of hydrogenase and RuBP carboxylase in these clones provides strong evidence of a single-point lesion.

Plants nodulated with the Hup^- PJ17 and PJ18 mutant strains lost H_2 from nodules, and bacteroid suspensions from nodules formed by these Hup^- mutants exhibited no measurable H_2 uptake capacity. These results (Table 3), in contrast to the observation with the Hup^+ parent strain which lost no H_2 from nodules, are consistent with previous research (9). The total N contents of plants inoculated with Hup^+ parent strain SR are convincingly higher than comparable values for plants inoculated with mutant strains and reflect the benefits of the H_2-oxidizing characteristic to the N_2-fixing process. Considerable biological variability among N contents and yields of replicate cultures was observed. Although the Leonard jar technique is excellent for bacteriological control, the rapid depletion of nutrients and the restricted root environment of the cultures introduces variables that make yield comparisons difficult. More controlled experiments are being conducted.

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