Rhizobium japonicum Mutant Strains Unable to Grow Chemoautotrophically with H₂†

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Rhizobium japonicum strain SR grows chemoautotrophically on a mineral salts medium when incubated in an H₂- and CO₂-containing atmosphere. Mutant strains unable to grow or that grow very poorly chemoautotrophically with H₂ have been isolated from strain SR. The mutant isolation procedure involved mutagenesis with ethyl methane sulfonate, penicillin selection under chemoautotrophic growth conditions, and plating of the survivors onto medium containing carbon. The resulting colonies were replica plated onto medium that did not contain carbon, and the plates were incubated in an H₂- and CO₂-containing atmosphere. Mutant strains unable to grow under these conditions were chosen. Over 100 mutant strains with defects in chemoautotrophic metabolism were obtained. The phenotypes of the mutants fall into various classes. These include strains unable to oxidize H₂ and strains deficient in CO₂ uptake. Some of the mutant strains were capable of oxidizing H₂ only when artificial electron acceptors were provided. Two mutant strains specifically lack activity of the key CO₂-fixing enzyme ribulose 1,5-bisphosphate carboxylase. Other mutant strains lack both H₂-oxidizing ability and ribulose 1,5-bisphosphate carboxylase activity.

Some strains of *Rhizobium japonicum* possess an H₂-uptake system both in free-living culture and as bacteroids isolated from soybean root nodules. The H₂-oxidation system is capable of recycling H₂ produced by the nitrogenase reaction. The oxidation of H₂ by bacteroids can provide ATP for N₂ fixation (7, 8), whereas H₂ oxidation by free-living *R. japonicum* can provide energy to support chemoautotrophic growth of the cells (9, 10). Greenhouse studies have been performed comparing the symbiotic effects of *R. japonicum* strains possessing H₂-uptake ability with strains lacking this ability. It was concluded that the possession by the *R. japonicum* strain of H₂-oxidizing ability results in significant increases in plant dry matter and in total N₂ fixed (1).

R. japonicum strains that express H₂-uptake activity as nodule bacteroids also can oxidize H₂ in free-living culture when subjected to the appropriate culture conditions (16). Expression of H₂-uptake activity in free-living cultures of *R. japonicum* (11, 16) enabled studies on factors affecting synthesis of this system (17). Oxygen and carbon substrates repress formation of the H₂-uptake system. H₂ and CO₂ are somehow involved in stimulation of the synthesis of the H₂-uptake system (17). Also, cyclic AMP may be involved in the regulation of synthesis of H₂-uptake-system components (12). Expression of

H₂ oxidation under free-living conditions also enabled the isolation of three H₂-uptake-negative (Hup⁻) mutants (18). These mutant strains were useful for the evaluation of benefits of the H₂-uptake system on N₂ fixation in soybeans (1).

Studies on the regulation of H₂ uptake in free-living *R. japonicum* indicated that CO₂ fixation activity was somehow correlated with H₂-oxidation activity (17, 24). Ribulose bisphosphate (RuBP) carboxylase was the enzyme primarily responsible for CO₂ fixation when *R. japonicum* cells were oxidizing H₂ (10, 24). RuBP carboxylase and H₂-uptake activities both were repressed by added carbon, and both uptake systems were induced when cells were incubated in an H₂- and CO₂-containing atmosphere. Since some *R. japonicum* strains showed the ability to utilize H₂ and CO₂, attempts were made to determine whether Hup⁺ *R. japonicum* strains could utilize H₂ as the sole source of energy while simultaneously using CO₂ as the carbon source. Autotrophic growth of some *R. japonicum* strains was subsequently demonstrated (9), and the culture conditions for such growth were improved (10). In addition, 3-phosphoglycerate was demonstrated as the product of the RuBP carboxylase reaction (10).

The hydrogenase system in bacteroids and free-living *R. japonicum* cells is complex, catalyzing the activation and oxidation of H₂ through a series of unidentified electron transport components. Oxygen is the terminal electron accep-

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tor, yielding water as the final product. No information is available concerning the specific components involved in the electron transport sequence between H_2 and O_2 . To determine these specific components, as well as to further investigate the regulation of hydrogen uptake in *R. japonicum*, many mutant strains lacking H_2 -uptake ability were needed. A relatively easy method to obtain such mutants, based on the inability of the strains to grow chemoautotrophically with H_2 , is described here. Characterization of the mutants indicates that most of them contain lesions in the H_2 -oxidation or CO_2 -fixation pathways.

MATERIALS AND METHODS

Chemicals. Kanamycin sulfate, streptomycin sulfate, Tris base, methylene blue, and phenazine methosulfate were obtained from Sigma Chemical Co., St. Louis, Mo. Ethyl methane sulfonate was obtained from Eastman Kodak Co., Rochester, N.Y. All other chemicals were of reagent grade and were obtained from Sigma Chemical Co. or J. T. Baker Chemical Co., Phillipsburg, N.J. $NaH^{14}CO_3$ of specific activity of 44.5 mCi/mmol was obtained from New England Nuclear Corp., Boston, Mass.

Media. The standard growth medium used was modified Bergersen medium (BM) as described by Bishop et al. (4). For autotrophic growth, this same medium was used, with the primary exceptions that no carbon was added, and NH_4Cl was added as the N source. The autotrophic growth medium contained (in 1 liter of distilled water): K_2HPO_4 , 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $MgCl_2 \cdot 6H_2O$, 0.51 g; NH_4Cl , 0.1 g; and the trace elements described by Bishop et al. for modified BM (4). This medium will be referred to as BM-no carbon. Other media used for growth of cells included H_2 -uptake medium (16).

Mutagenesis and penicillin selection. The isolation of *R. japonicum* strain SR has been described previously (18). This was the wild-type strain used for all studies. The ethyl methane sulfonate mutagenesis procedure used for *R. japonicum* strain SR was similar to that described by Meade and Signer for the mutagenesis of *Rhizobium meliloti* (19). *R. japonicum* strain SR was grown in BM to stationary phase (optical density at 540 nm of 1.5). A total of 100 ml of the culture was harvested by centrifugation at $10,400 \times g$ for 15 min, and the pellet was suspended in 2 ml of 0.2 M Tris (pH 7.6) containing 0.85% NaCl (20). Ethyl methane sulfonate (0.05 ml) was added, and the suspension was incubated for 1 h at 30°C. Then the suspension was centrifuged at $10,800 \times g$ for 10 min, and the pellet was washed twice with 20 mM $Na_2S_2O_3$ (19). This procedure gave between 88.4 and 93.2% kill in separate mutagenesis experiments. The mutagenized cells were suspended in yeast extract-mannitol medium (27) and grown at 30°C for 3 days (to an optical density at 540 nm of 1.2). After centrifuging this suspension at $10,800 \times g$ for 10 min, the pellet was washed in BM-no carbon to an optical density at 540 nm of 0.4. The cells were placed into sterile 250-ml centrifuge bottles, which were fitted loosely with caps

and then placed into a gas-tight container. The gas-tight container was flushed with N_2 until no O_2 could be detected amperometrically and then flushed with a gas mixture composed of 85% N_2 , 10% H_2 , and 5% CO_2 . Oxygen was injected to attain a final O_2 concentration of 1%. The container was shaken (120 cycles per min) at 30°C for 3 days. During this time, gas samples were removed by syringe, and O_2 and H_2 were monitored amperometrically. Gases were injected to maintain O_2 near 1% and H_2 near 10%. After 3 days under autotrophic growth conditions, penicillin was added (10,000 U/ml of culture), and the cells were reincubated in an atmosphere composed of 84% N_2 , 10% H_2 , 5% CO_2 , and 1% for 6 h. The cells were harvested by centrifugation and washed three times with 0.85% NaCl, and dilutions were plated onto BM. Plates were incubated at 30°C for 6 to 10 days, and colonies were replica plated onto BM-no carbon. The BM-no carbon plates were incubated for 9 to 12 days in an atmosphere of 84% N_2 , 10% H_2 , 5% CO_2 , and 1% O_2 . The containers were refushed with the N_2 - H_2 - CO_2 mixture every 3 days, and H_2 and O_2 were injected by syringe to maintain the H_2 and O_2 concentrations at 10% and 1%, respectively, as monitored amperometrically. Mutant strains unable to grow under these conditions were chosen and retested for inability to grow chemoautotrophically with H_2 . The mutant strains were also tested for inability to grow on the mineral salts medium described by Hanus et al. (9), in the H_2 - and CO_2 -containing atmosphere.

Since the original plating medium (BM) contained glutamate as the N source, whereas the mutant screening medium contained NH_4Cl , some mutant strains might be expected to be glutamate auxotrophs. All of the mutant strains were tested for ability to grow on BM containing 0.1 g of NH_4Cl per liter rather than glutamate as N source. Mutant strains exhibiting poor growth on this medium were discarded. These amounted to about 6% of the total original "mutant" population.

H_2 -uptake derepression and H_2 -uptake assay. *R. japonicum* strain SR or mutant strains derived from strain SR were derepressed for H_2 -uptake activity by suspending cells in 0.05 M potassium phosphate buffer (pH 7.0) containing 2.5 mM $MgCl_2$, as previously described (17). Small volumes of cells (15 ml of approximately 5×10^8 cells per ml) suspended in the buffer were added to 250-ml prescription bottles; the bottles were sealed and flushed with N_2 , and sufficient H_2 , CO_2 , and O_2 were added to obtain an atmosphere composed of 84% N_2 , 10% H_2 , 5% CO_2 , and 1% O_2 . The bottles were incubated at 30°C and shaken at 120 cycles per min. H_2 and O_2 concentrations were monitored amperometrically and maintained at 10% and 1%, respectively. Unless otherwise stated, the derepression period was 40 h. Rates of H_2 uptake were measured amperometrically as described (17, 28), except that the amperometric chamber volume was 4.5 ml. Cell numbers were estimated by comparison of optical density (540 nm) measurements with a standard curve of viable cell number versus optical density at 540 nm.

Whole cell $^{14}CO_2$ uptake. Whole cell $^{14}CO_2$ uptake was measured as described in Table 2, footnote a. Counts obtained from reaction vials containing boiled

cells of each strain were subtracted from counts per minute values for the corresponding strain.

RuBP carboxylase activity in permeabilized whole cells. RuBP carboxylase was measured by determining the rate of $^{14}\text{CO}_2$ fixation into acid-stable products. This was determined in toluene-treated cells by a procedure similar to that reported by Tabita et al. (26) and Anderson (2). The validity of this assay as an accurate measurement of cell-free RuBP carboxylase activity was demonstrated by Tabita et al. (26). The RuBP carboxylase activity obtained in toluene-treated photosynthetic bacteria approximately the activity in cell-free extracts (26). This permeabilized whole cell assay has also been used to measure RuBP carboxylase activity in *R. japonicum* strain SR (10). The cells from 10 ml of derepressed culture were harvested and suspended in 0.3 ml of 0.05 M Tris-hydrochloride (pH 7.8) containing 0.025 M NaHCO_3 , 0.01 M MgCl_2 , and 0.001 M EDTA (2). The 0.3-ml suspension was transferred to a test tube, the test tube was sealed with a serum stopper, and 20 μl of toluene was injected by use of a syringe. The cell suspension-toluene mixture was gently mixed for 2 min and then allowed to incubate for 10 min at 25°C to allow activation of RuBP carboxylase (2, 13, 26). Three 50- μl portions were taken from each toluene-treated sample for assay of RuBP carboxylase. The 50- μl cell sample was added to 0.1 ml of freshly prepared assay mixture (0.05 M Tris-hydrochloride [pH 7.8], 0.01 M MgCl_2 , 0.001 M EDTA, 1.5 mM RuBP, 25 mM $\text{NaH}^{14}\text{CO}_3$ [0.4 Ci/mol]) in a stoppered test tube (2). The reactions proceeded for 20 min at 30°C and were stopped by the addition of 0.2 ml of 4 N HCl. Under these conditions, the reaction was linear during the 20-min time period, and the specific activity was proportional to the volume of cell suspension added. Total cell protein was determined after boiling cells in NaOH as described by Stickland (25). The dye-binding method of Bradford (6) was used for quantification of protein (protein assay kit; Bio-Rad Laboratories, Richmond, Calif.) with bovine gamma globulin as the standard. Radioactivity was determined by use of a Packard 3375 liquid scintillation spectrometer. Disintegration per minute was determined from counting efficiencies ranging from 0.80 to 0.87. Reactions containing cells that had been boiled after derepression, plus RuBP and $\text{NaH}^{14}\text{CO}_3$, gave radioactivities of 25 to 38 cpm. Control reactions were conducted in which substrate (RuBP) or cells were omitted. The values reported in Table 3 were obtained by subtracting the counts per minute value obtained for the endogenous (no RuBP added) from that of samples of the same strain containing added RuBP.

Cell-free RuBP carboxylase activity. Cell-free RuBP carboxylase was determined by the method of Wishnick and Lane (29), exactly as described previously by Simpson et al. (24). Derepressed cells were disrupted by passing twice through a French pressure cell at 15,000 lb/in², and extracts were prepared by centrifugation at 15,000 $\times g$ for 30 min. The supernatant liquid contained approximately 0.5 mg of protein and was assayed for RuBP-dependent CO_2 fixation. Control reactions were conducted in which RuBP or cell-free extracts were omitted. Units of RuBP-dependent CO_2 uptake were determined for direct com-

parison with activities in toluene-treated cells. All other conditions were identical to that described by Simpson et al. (24), with the exception that protein was estimated by the dye-binding method of Bradford (6) and a Packard 3375 liquid scintillation spectrometer was used. Counting efficiencies ranged from 0.80 to 0.87.

RESULTS

After ethyl methane sulfonate mutagenesis followed by penicillin selection under autotrophic growth conditions, approximately 8.5×10^4 individual colonies were replica plated and screened for inability to grow on a mineral salts medium in an H_2 - and CO_2 -containing atmosphere. Over 100 mutants with defects in autotrophic metabolism have thus far been obtained. All of the mutant strains grew well on heterotrophic (BM) medium. All of the mutant strains contained both of the antibiotic resistance characteristics (streptomycin and kanamycin resistance) of the parent strain, insuring that they are not contaminants. The parent strain SR and the mutants discussed here were tested for nodulation of soybeans by the pouch method as described (15). All of these strains nodulated soybeans. All of the mutant strains analyzed for H_2 oxidation and CO_2 fixation that are unable to grow or that grow very poorly chemoautotrophically with H_2 have been found to contain defects in either the H_2 - or the CO_2 -uptake system or both. It is important to note that the original plating medium contained glutamate as N source, whereas the mutant screening medium contained NH_4Cl . Consequently, some of the mutants obtained were glutamate auxotrophs. These strains were eliminated before further characterization of the mutant strains (see above).

Growth of the parent strain SR and mutants chemoautotrophically with H_2 . Hanus et al. (9) demonstrated that *R. japonicum* strains possessing H_2 -uptake ability were capable of growth in a mineral salts medium, in an H_2 - and CO_2 -containing atmosphere. For autotrophic growth, a medium similar to that described by Hanus et al. (9) was used (see above for description of media). All of the mutant strains were tested for ability to grow on both the agar medium described by Hanus et al. (9) and the medium described in the text when incubated in an H_2 - and CO_2 -containing atmosphere. The mutant strains grew poorly or not at all on both media, whereas strain SR showed considerable growth.

H_2 uptake with artificial acceptors. Most of the mutant strains unable to grow chemoautotrophically were unable to oxidize H_2 when tested amperometrically with oxygen provided

as electron acceptor. The phenotypic designation Hup^- , originally given to strains of this phenotype (18), has also been given to the strains described here that are unable to oxidize H_2 . Since the Hup^- mutant strains lacked the ability to catalyze the oxyhydrogen reaction, their specific defect could be due to a deficiency in one or more of the unknown electron carriers that function between H_2 and O_2 . Therefore, the addition of artificial electron acceptors to cell suspensions of the mutants might restore H_2 -uptake capability by accepting electrons at some point in the electron transport chain before the presumed lesion. Methylene blue is capable of accepting electrons from purified *R. japonicum* bacteroid hydrogenase (3). Therefore, Hup^- strains that still retain the ability to synthesize the H_2 -activating enzyme hydrogenase should be able to take up H_2 when methylene blue is provided as an electron acceptor.

Hup^- strains were tested for their ability to oxidize H_2 when the artificial electron acceptors methylene blue and phenazine methosulfate were provided. As shown in Table 1, the mutant strains were unable to oxidize H_2 when O_2 was provided as terminal electron acceptor. However, strains SR106 and SR166 were able to oxidize H_2 when the artificial electron acceptors were provided. The wild-type strain SR also can oxidize H_2 with these acceptors in the absence of oxygen (Table 1). In these experiments, the cultures were derepressed for H_2 uptake in phos-

phate buffer under an atmosphere composed of 84% N_2 , 1% O_2 , 10% H_2 , and 5% CO_2 . Since this medium contains no oxidizable organic substrates and the mutant strains lacked the ability to oxidize H_2 , the mutant strains were energy deficient. Therefore, the fact that the methylene blue-dependent H_2 -uptake activity of the wild type was greater than that of the Hup^- mutants SR106 and SR166 could be a result of the inability to the mutants to provide sufficient energy for maximal protein synthesis during the derepression period. Most of the Hup^- strains tested (approximately 50) did not oxidize H_2 when methylene blue was provided as an acceptor (for example, SR119 in Table 1). Only three mutants have been obtained that exhibit a phenotype like SR106 and SR166. This indicates that this class of mutant is relatively rare. Strains SR106 and SR166 apparently retain the initial component(s) (presumably the H_2 -activating hydrogenase) required in the H_2 -uptake pathway and may lack electron transport factors normally operating between the H_2 -activating hydrogenase and the terminal electron acceptor oxygen.

H_2 -uptake and CO_2 -fixation activities in mutant strains. Preliminary experiments were performed to localize the defects in various mutants in either the H_2 - or CO_2 -uptake systems. Most (approximately 70%) of the mutant strains tested were unable to oxidize H_2 . To determine the total CO_2 -fixation capability of mutant strains, CO_2 -uptake rates were measured on H_2 -uptake-derepressed whole cells of strain SR and various mutant strains. The results for some strains are shown in Table 2. All mutant strains unable to oxidize H_2 (for example, strains SR118, SR114, and SR166 in Table 2) had low CO_2 -fixation activities. Perhaps this observation is a result of some of the Hup^- mutant strains inability to oxidize H_2 and therefore, their inability to provide ATP for carboxylation reactions or for synthesis of carboxylation enzymes. Some mutant strains unable to grow chemoautotrophically with H_2 were deficient in whole cell CO_2 fixation even though they were capable of oxidizing H_2 . For example, mutant strains SR173, SR122, SR141, and SR147 (Table 2) had CO_2 -fixation rates much less than the wild-type strain SR, even though these strains were able to oxidize H_2 . Mutant strains of this type were later analyzed for the presence of the key CO_2 -fixing enzyme RuBP carboxylase (see below).

RuBP carboxylase activity in mutant strains. Autotrophic growth of *R. japonicum* strain SR and other H_2 -oxidizing *R. japonicum* strains has been previously demonstrated (9, 10). RuBP carboxylase and propionyl coenzyme A carboxylase were detected in H_2 -uptake-dere-

TABLE 1. H_2 uptake of Hup^- *R. japonicum* strains with O_2 , methylene blue, and phenazine methosulfate as electron acceptors^a

Strain	H_2 uptake activity (nmol/h per 10^8 cells)		
	O_2 (0.02 mM)	MB (1.0 mM)	PMS (5.0 mM)
SR	59.1	13.2	18.3
SR106	<0.5	3.2	3.6
SR166	<0.5	3.7	4.0
SR119	<0.5	<0.5	<0.5

^a Cultures were derepressed for 40 h in 0.05 M potassium phosphate buffer (pH 7.0) containing 2.5 mM MgCl_2 in an atmosphere composed of 84% N_2 , 10% H_2 , 5% CO_2 , and 1% O_2 as described in the text. The cultures were then flushed with N_2 and added to the 4.5-ml amperometric chamber for determination of H_2 -uptake rates as described (15, 24). After adding 37.7 nmol of H_2 (as a saturated solution) and the appropriate electron acceptor at the concentration indicated, H_2 -uptake rates were determined. Saturating concentrations of the electron acceptors were used, and methylene blue (MB)- and phenazine methosulfate (PMS)-dependent H_2 uptake was determined in the absence of O_2 . Cell concentrations were approximately 5×10^8 cells/ml.

pressed *R. japonicum* (24), and it was concluded that RuBP carboxylase was the primary carboxylase operating during autotrophic metabolism (10). Therefore, some of the mutant strains obtained might be expected to lack a key enzyme (in particular, RuBP carboxylase) of the autotrophic CO₂-fixation pathway. Toluene-treated cells of various mutant strains were assayed for RuBP carboxylase activity, and the results are shown in Table 3. Some mutant strains possessing H₂-oxidizing ability, for example, SR122 and

TABLE 2. H₂-uptake and CO₂-uptake rates of whole cells of strain SR and various mutant strains

Strain	H ₂ uptake ^a (nmol/h per 10 ⁸ cells)	CO ₂ uptake nmol/ min per mg of pro- tein)
SR	95.2	7.6 ± 0.6
SR173	56.8	<0.5
SR122	65.4	<0.5
SR141	78.8	<2.0
SR147	73.6	<2.0
SR118	<0.5	<0.5
SR166	<0.5	<0.5
SR114	<0.5	<0.5

^a Each reported value is the average of two independent amperometric determinations (see the text). Cells were derepressed for H₂ uptake in an H₂- and CO₂-containing atmosphere for 40 h as described in the text. Derepressed cells (5 ml of 5 × 10⁸ cells/ml) were added to a 32-ml serum bottle; the bottle was flushed with N₂ and sealed with a serum stopper, and gases were injected to obtain an atmosphere of 89% N₂, 10% H₂, and 1.0% O₂. Sufficient NaHCO₃ was added to obtain a reaction concentration of 20 mM. The final reaction solution contained 0.1 μCi of NaH¹⁴CO₃ per μmol. The sealed vials were shaken at 30°C for 30 min, and three 1-ml samples were removed from the 5-ml reaction volume for assay of radioactivity. The 1-ml samples were added to scintillation vials containing 0.3 ml of 60% trichloroacetic acid; the vials were shaken and left 48 h in a fume hood. This procedure was described by Lepo et al. (10). One-milliliter samples of cells were also filtered onto Whatman GF/F filter disks, the cells on the disks were washed, and the disks were dried and counted to compare activities with acid-treated samples. The radioactivities of samples treated in this manner were comparable to acid-treated samples. Therefore, the acid treatment and the 48-h incubation in the fume hood were found to be sufficient to dissipate most of the ¹⁴CO₂ that was not fixed. Ten milliliters of Aquasol (New England Nuclear Corp.) was added to the vials containing cells, and the counting rate during a 10-min period was determined. Counts obtained from reaction vials containing boiled cells of each strain after derepression were subtracted from counts per minute values for the corresponding strain. Vials containing boiled cells had counts ranging from 55 to 95 cpm. The specific activities of three replicate samples for each strain were determined. The reported value for strain SR is the mean ± the standard deviation for the three replicate samples.

TABLE 3. H₂-uptake and RuBP carboxylase activities in strain SR and various mutant strains^a

Strain	H ₂ uptake (nmol/h per 10 ⁸ cells)	RuBP carboxylase (nmol/min per mg of protein)
SR	77.9	9.3 ± 0.9
SR141	67.7	6.6 ± 0.8
SR147	74.5	8.1 ± 0.6
SR173	59.3	<1.0
SR122	54.2	<1.0
SR140	<0.5	<1.0
SR163	<0.5	<1.0
SR106	<0.5	<1.0

^a H₂-uptake values are the average of two independent amperometric determinations on cells derepressed as described for 40 h. For assay of RuBP carboxylase, the cells were subjected to derepression conditions for 40 h, toluene-treated, and assayed for RuBP-dependent CO₂ fixation into acid-stable products as described in the text. Control carboxylase assays which lacked added RuBP were conducted for each strain. The values reported above were obtained by subtracting the counts per minute values for the endogenous (no RuBP added) value from those of samples of the same strain containing added RuBP. Reactions containing cells that had been boiled after derepression, plus RuBP and NaH¹⁴CO₃, gave radioactivities of 25 to 38 cpm. The specific activity of three replicate samples for each strain was determined. The reported values for those strains containing activity (SR, SR141, SR147) is the mean ± the standard deviation for the three replicate examples.

SR173 in Table 3, lacked RuBP carboxylase activity. These two mutant strains also had consistently lower H₂-uptake activity than did the parent strain SR (Tables 2 and 3). Strains SR173 and SR122 were later analyzed for cell-free RuBP carboxylase activity (see below). Other H₂-oxidizing mutant strains have been obtained which are able to oxidize H₂, contain RuBP carboxylase activity, and yet are deficient in whole cell CO₂ fixation. Examples are strain SR141 and SR147 (Tables 2 and 3). These types of mutant strains may have deficiencies in other enzymes involved in CO₂ metabolism.

Although the validity of toluene treatment for the assay of RuBP carboxylase is well documented for photosynthetic bacteria (26), it is important to be certain that this assay approximates the activity observed in cell-free extracts of *R. japonicum* SR. Cell-free extracts of derepressed strain SR and some of the mutants (SR173 and SR122) were assayed for RuBP-dependent CO₂ fixation (as described above). The total units of RuBP-dependent CO₂ fixation in cell-free extracts of derepressed strain SR was 120% of that observed in toluene-treated cells. Therefore, the toluene-treated cell assay approximates the activity of cell-free extracts. Cell-free extracts of mutant strains SR173 and SR122 did

not have RuBP carboxylase activity; therefore, these strains are unequivocally lacking RuBP carboxylase activity.

The induction kinetics of RuBP carboxylase parallel that of H₂-uptake activity in H₂-uptake-derepressed *R. japonicum* strain SR (24). Also, both uptake systems are repressed by the addition of succinate (24). To investigate this apparent regulatory relationship between the two uptake systems, many mutant strains lacking H₂-oxidizing ability were assayed also for RuBP carboxylase activity. All Hup⁻ strains (30 strains were tested) had negligible (<1.0 nmol/min per mg of protein) RuBP carboxylase activity. Examples of such strains are SR140, SR163, and SR106 in Table 3. In these experiments, the cultures were derepressed for H₂ uptake and RuBP carboxylase in phosphate buffer under an atmosphere composed of 84% N₂, 1% O₂, 10% H₂, and 5% CO₂. Since this medium contains no oxidizable organic substrates and the mutant strains lacked the ability to oxidize H₂, the strains were probably energy deficient. Therefore, the low or negligible RuBP carboxylase activity could be due to a lack of ATP to synthesize RuBP carboxylase, rather than a specific defect in RuBP carboxylase-related genes. Many attempts were made to obtain RuBP carboxylase activity that was not dependent on the energy-yielding H₂-oxidation pathway. Carbon substrates were added to previously derepressed cultures of mutants at various times during derepression in an attempt to provide energy for RuBP carboxylase formation. Some RuBP-dependent CO₂ fixation was observed with some mutant strains in some experiments when succinate (30 mM) was provided 4 h before assay. However, the amount of stimulation by succinate was not consistent, and most values were still less than 1.0 nmol/min per mg of protein. This activity is not sufficient to conclude that RuBP-dependent fixation was occurring in these strains. Therefore, the capability of the Hup⁻ mutants to express RuBP carboxylase activity remains unclear. The difficulty of this approach (addition of a carbon source to observe RuBP carboxylase) undoubtedly is due to the well-known repression of RuBP carboxylase by carbon sources in chemoautotrophic bacteria.

Revertant studies. Spontaneous revertants of some mutant strains were obtained by plating mutants on the no carbon-mineral salts medium and choosing colonies that were capable of growth in the H₂- and CO₂-containing atmosphere. Mutant strains that were specifically lacking RuBP carboxylase activity (SR173 and SR122) were easily revertible (spontaneous reversion frequencies of 3.5×10^{-7} and 3.9×10^{-7} ,

respectively). Although revertants have been obtained from some Hup⁻ mutant strains (such as SR106 and SR118), revertants have not been obtained from some other mutant strains lacking H₂-uptake ability. These mutants may have lost either a plasmid or chromosomal DNA carrying the information for H₂-oxidation components. Alternatively, the nonreverting strains may contain multiple lesions. Biochemical and genetic studies are needed to explain the observed phenotype of these mutant strains.

DISCUSSION

Over 100 *R. japonicum* mutant strains unable to grow chemoautotrophically with H₂ have been obtained. The mutant isolation procedure involved mutagenesis of the wild type with ethyl methane sulfonate, penicillin selection under autotrophic growth conditions, plating of the survivors onto medium containing carbon, and replica plating of the resulting colonies onto medium containing no carbon. Many of these mutant strains have been screened for their capacity to oxidize H₂ or fix CO₂. In all cases, after eliminating glutamate auxotrophs, the inability of mutant strains to grow chemoautotrophically with H₂ can be correlated to a deficiency in the ability either to oxidize H₂ or to incorporate CO₂. Therefore, the mutant screening method described is useful for the isolation of mutants specific to chemoautotrophic metabolism. Although Hup⁻ mutant strains of *R. japonicum* were obtained previously (18), the mutant screening procedure was time consuming and laborious and utilized bacteroids rather than free-living cells as the starting material. In contrast, the procedure described here involving penicillin selection, replica plating, and screening for inability of colonies to grow chemoautotrophically with H₂ enables the rapid isolation of many such mutant strains. This type of approach (analysis of chemoautotrophic-growth-negative mutants) has been used to study the regulation of H₂-oxidation and CO₂-fixation systems in the autotrophic H₂-oxidizing bacterium *Alcaligenes eutrophus* (2, 22).

Analysis of approximately 50 chemoautotrophic-growth-negative mutant strains has revealed several different classes of mutants containing defects in chemoautotrophic metabolism. These classes include mutants unable to oxidize H₂, strains deficient only in CO₂ uptake, and strains lacking RuBP carboxylase activity.

One large group of mutants is unable to carry out the oxyhydrogen reaction. In this group are mutant strains that can oxidize H₂ when provided with artificial electron acceptors. These

strains presumably contain the initial H₂-activating hydrogenase and may lack other electron-transporting components normally operating between H₂ and O₂. Biochemical characterization of mutants specifically unable to oxidize H₂ will provide information on the number and nature of the electron transport factors operating between H₂ and O₂. Currently, the number and type of electron transport components operating in this system are unknown.

Other classes of mutants are useful for understanding the regulation of H₂ uptake in conjunction with CO₂ fixation via RuBP carboxylase. The regulation of synthesis of H₂-uptake-pathway and CO₂-fixation components in *R. japonicum* is complex and not well understood. Carbon dioxide is somehow involved in stimulation of the synthesis of H₂-uptake-system components in *R. japonicum* (17). Stimulation of H₂ uptake by added CO₂ has also been observed in some autotrophic H₂-oxidizing bacteria (5, 22, 23). Coordinate induction of H₂ uptake and RuBP carboxylase in *R. japonicum* was reported (24). One class of mutants isolated here is able to oxidize H₂ but lacks RuBP carboxylase activity. Both toluene-treated cells and cell-free extracts of these mutants lacked measurable RuBP carboxylase activity. These strains also had lower H₂-uptake activity than did the parent type. This result indicates the existence of a regulatory relationship between CO₂-fixation and maximum H₂-uptake rates.

All mutant strains unable to oxidize H₂ also had negligible RuBP carboxylase activity. This result agrees with the results obtained for previously isolated Hup⁻ *R. japonicum* mutant strains (24) and supports the previous suggestion (17, 24) that a coordinate regulatory relationship exists between the two uptake systems. Alternatively, the RuBP carboxylase activity of *R. japonicum* strain SR is low, and perhaps some measurable activity could be obtained in some Hup⁻ strains under appropriate culture conditions. Since the Hup⁻ mutant strains were incubated in a medium that lacked oxidizable carbon substrates, it is also possible that small amounts of RuBP carboxylase were proteolytically degraded during the 40-h derepression (starvation) conditions. It is interesting to note that some Hup⁻ mutants of *A. eutrophus* isolated by Anderson (2) still retained low levels of RuBP carboxylase activity. Further analysis of mutants specifically lacking RuBP carboxylase or H₂-uptake-system components should provide information on the coordinate regulation of these two uptake systems.

Although the mutant strains described here form effective nodules on soybean plants, other

symbiotic properties have not yet been examined. The symbiotic properties of previously isolated Hup⁻ mutant strains indicated that the possession of H₂-uptake ability has beneficial effects on N₂ fixation (1). Soybeans inoculated with the Hup⁺ parent strain had greater nitrogen-fixation rates, dry weight, and N content than did plants inoculated with the Hup⁻ mutant strains. Similar experiments with carefully chosen Hup⁻ strains described here can confirm and extend these observations.

The symbiotic properties of *R. japonicum* strains specifically lacking RuBP carboxylase have not been examined. Various carboxylation enzymes are present in the nodule cytosol and in bacteroids isolated from nodules (14, 21). Simpson et al. (24) could not detect RuBP-dependent CO₂ fixation in bacteroids of *R. japonicum* strain SR. If RuBP carboxylase is not expressed in nodule bacteroids, then mutant strains lacking this enzyme should have normal symbiotic properties. The symbiotic characteristics (N₂ fixation and H₂ uptake) of various mutant strains deficient in free-living CO₂ fixation will therefore be interesting.

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