Chemoautotrophic Growth of Hydrogen-Uptake-Positive Strains of *Rhizobium japonicum*

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Recently reported research from this laboratory has demonstrated the autotrophic growth of certain hydrogen-uptake-positive strains of *Rhizobium japonicum* and defined minimal conditions for such growth. Ribulose 1,5-bisphosphate carboxylase has been detected in autotrophically growing cells, but at low specific activity. Moreover, growth rates were low, and growth ceased at low cell densities. We report here improved autotrophic growth rates of *R. japonicum* SR through the use of a modified mineral salts/vitamins medium and a programmed increase in oxygen tension as autotrophic growth proceeds. Under these conditions, ribulose 1,5-bisphosphate carboxylase activity increased greater than 10-fold and crude-extract-uptake–hydrogenase activities were from 20 to 47 times those heretofore reported for free-living *R. japonicum*. It is likely that previous assays for these enzymes were done on preparations of cells in which their synthesis had been partially repressed. The contribution of CO_2 fixation to organic carbon accumulation in autotrophic cells was assessed as sufficient to support observed growth. Enzymological determination of the product of carbon fixation has established a stoichiometric ratio of 1.9 mol of 3-phosphoglycerate per mol of CO_2 fixed and unequivocally assigns the role of carbon fixation catalysis to ribulose 1,5-bisphosphate carboxylase. Ammonium served best as a nitrogen source, nitrate was less effective, and gaseous nitrogen would not support autotrophic growth. Ecological, evolutionary, and practical considerations of autotrophy in the rhizobia are briefly discussed in the light of our findings.

The autotrophic growth of some strains of *Rhizobium japonicum* has been recently reported by Hanus et al. (8). Chemolithotrophic growth occurred only in those strains possessing hydrogen uptake capacity and under an atmosphere of low-oxygen partial pressure, but of enriched hydrogen and carbon dioxide content. Subsequently, propionyl coenzyme A carboxylase and ribulose 1,5-bisphosphate (RuBP) carboxylase activities in cells derepressed for uptake hydrogenase activity were determined by Simpson et al. (19). RuBP carboxylase and uptake hydrogenase were coordinately induced by H_2 in the gas phase and were both repressed by succinate. Maier et al. (12) have reported that oxygen concentration in the gas phase must be kept at 1% to ensure derepression of hydrogenase synthesis and that a variety of organic compounds supplied in the media completely repressed hydrogenase synthesis (12, 13).

In these early investigations, autotrophic growth rates of *R. japonicum* were low, and growth ceased at low cell population densities (J. E. Lepo, unpublished data; absorbancy at 540 nm of less than 0.3). Moreover, RuBP carboxylase activities were low when compared with those of other bacteria capable of hydrogen-supported chemolithotrophic growth (19).

Therefore an investigation was initiated to improve autotrophic growth rates of *R. japonicum*, to further define requirements for and characteristics of autotrophic growth, and to determine the extent to which CO_2 fixation via RuBP carboxylase might be involved in such growth. Through the use of a modified medium and programmed increase in oxygen tension, we have obtained higher growth rates and a 10-fold increase in specific activity of RuBP carboxylase over that reported by Simpson et al. (19). We show that $^{14}CO_2$ assimilation rates during growth are sufficient to account for most of the carbon that accumulated, and that RuBP carboxylase activity of three hydrogenase-positive *R. japonicum* strains is more than adequate to account for all the carbon fixed. Moreover, enzymological assay shows the product of carbon fixation to be nearly 2 mol of 3-phosphoglycerate for each mol of CO_2 fixed.

Prior to this study, the capacity of *Rhizobium* spp. to recycle hydrogen via the unidirectional uptake hydrogenase was most efficiently elicited by growing the organisms on a low-carbon medium and then derepressing in a carbon-free buffer under an atmosphere containing hydrogen and low partial pressure of oxygen (13). Hydrogen uptake rates of our autotrophically

grown cells are greatly enhanced over rates heretofore obtained by other means of derepression. The effect of nitrogen source was also determined.

MATERIALS AND METHODS

Chemicals. NaH^{14}CO$_3$, of specific activity of 48 mCi/mmol, was obtained from New England Nuclear Corp., Boston, Mass. Glyceraldehyde 3-phosphate dehydrogenase, 3-phosphoglyceric phosphokinase, and triosephosphate isomerase were purchased from Sigma Chemical Co., St. Louis, Mo., and α-glycerophosphate dehydrogenase was obtained from Calbiochem-Behring Corp., La Jolla, Calif. Other chemicals were purchased from Sigma and from J. T. Baker Chemical Co., Phillipsburg, N.J. Gases were purchased from Airco Industrial Gases Div., Vancouver, Wash., and were of the highest purity available.

Strains. R. japonicum SR is a spontaneous streptomycin (250 μg/ml) and kanamycin (100 μg/ml)-resistant mutant of R. japonicum USDA DES 122. Strains USDA 136 and 3I1b6 of R. japonicum were obtained from Deane Weber of the U.S. Department of Agriculture.

Media and culture conditions. The mineral salts/vitamins medium of Madigan and Gest (11) was adapted for use as the autotrophic growth medium as follows: pH was adjusted to 7.0; FeSO$_4$, ethylenedenitrilotetraacetic acid (disodium salt), and phosphate stocks (pH 7.0) were added aseptically after other components of the medium had been autoclaved and cooled. Solid media were made by addition of 1.5% Noble agar (Difco Laboratories, Detroit, Mich.).

Autotrophic cultures of R. japonicum were grown in 125-ml prescription bottles, each containing 50 ml of medium. These were placed inside 3.8-liter glass jars having lids fitted with serum vial stoppers to facilitate adjustment of the gas mixtures. The composition of the atmosphere of 1% O$_2$, 5% CO$_2$, 10% H$_2$, and 84% N$_2$ was monitored daily by using a Hewlett-Packard 5830A gas chromatograph as described previously (12) and adjusted as necessary.

Inocula for autotrophic growth experiments were 1% of an autotrophically grown culture with an optical density at 540 nm of 0.2 to 0.3.

H$_2$ uptake rates. Hydrogen uptake rates were measured amperometrically as described (8, 14, 22) in the presence of saturating concentrations of H$_2$ and O$_2$.

RuBP carboxylase assay. RuBP carboxylase was determined by the method of McFadden et al. (15), and whole-cell assays were done at 30°C on cells treated with toluene as described by Tabita et al. (21). Extract prepared as described by McFadden et al. (15), or toluene-treated cells (50 μl) were preincubated for 5 min at 30°C in a mixture of: 100 μl of 160 mM Tris-hydrochloride (pH 8.0) with 50 mM MgCl$_2$ and 50 μl of 100 mM NaH^{14}CO$_3$. The reaction was initiated with 50 μl of 1.6 mM tetrasodium RuBP; the final reaction volume was 0.25 ml. Reaction mixtures without RuBP and without enzyme were included as controls, and radioactivity was determined as described below.

The stoichiometry of the product formed by RuBP

carboxylase was established by determining the amount of 3-phosphoglycerate produced. 3-Phosphoglycerate was determined by converting it to α-glycerophosphate by the action of the following coupled enzymes: 3-phosphoglycerate phosphokinase, glyceraldehyde 3-phosphate dehydrogenase, triose phosphate isomerase, and α-phosphoglycerate dehydrogenase in the presence of ATP, Mg^{2+}, and NADH. The decrease in absorbance at 340 nm due to oxidation of NADH was used to compute the amount of phosphoglycerate; 2 mol of NADH is oxidized per mol of phosphoglycerate (5).

Organic carbon determinations. Carbon analyses were performed on 12.5-ml samples of cells and medium at times during growth as indicated in Results. The method of Seargent and Reiman (18) was modified such that optical path and reaction volume were reduced by half; glucose was used as a standard. The absorbancy at 610 nm resulting from reaction of inoculated medium (time 0) was subtracted from that of subsequent determinations to estimate accumulation of organic carbon by autotrophic cultures of R. japonicum.

Determination of ^{14}CO$_2$ uptake rates. To verify that CO$_2$ uptake rates could sufficiently account for organic carbon accumulation, experiments in which ^{14}CO$_2$ was assimilated under conditions similar to those of growth were performed.

Radiolabeled CO$_2$ was generated as follows. Dry carrier NaHCO$_3$ (0.5 mmol) was placed into a serum bottle (40-ml nominal volume); the bottle was stoppered and evacuated. A solution with 50 μCi of NaH^{14}CO$_3$ (1.11 × 10^8 dpm) was injected into the bottle. This was sufficient label for four ^{14}CO$_2$ uptake assays. Concentrated H$_2$SO$_4$ (0.5 ml) was then injected, and CO$_2$ was allowed to accumulate. The gas was equilibrated to atmospheric pressure by allowing acidified (<pH 1.0 with H$_2$SO$_4$), degassed water to flow from a 100-ml graduated cylinder into the serum bottle via a Tygon hose fitted with a hypodermic needle. Thus the volume of the generated gas phase at atmospheric pressure was easily measured, and the specific radioactivity was calculated.

Culture bottles were removed from the incubation jars, the screw caps were removed, and the bottles were immediately fitted with fold-over serum vial stoppers. Gas mixtures in these bottles were monitored chromatographically and adjusted if necessary to 1% O$_2$, 5% CO$_2$, 10% H$_2$, and 84% N$_2$. One-quarter of the head-space volume from the serum bottle in which ^{14}CO$_2$ was generated was injected into each culture bottle to be used for an assay (2.775 × 10^7 dpm total per assay).

These cultures were incubated under growth conditions, and 1-ml samples were transferred to scintillation vials (using a 1-ml tuberculin syringe) at time 0 and hourly for 4 h thereafter. The samples were acidified with 0.3 ml of 60% trichloroacetic acid, and CO$_2$ that had not been fixed was allowed to dissipate overnight into a fume hood. Fifteen milliliters of scintillation cocktail (930 ml of scintillation-grade toluene, 4.0 g of 2,5-diphenyloxazole, and 70 ml of Beckman Biosolve) was added to each vial, and radioactivity was determined by use of a Packard 2425 scintillation spectrometer. Rates of CO$_2$ uptake that were linear

with time were obtained after equilibration of $^{14}CO_2$ with the liquid phase (about 1 h); these remained linear for at least 2 h.

Protein determinations. Cells were removed from samples of culture medium by centrifugation, and the pellets were suspended in 2 ml of 0.05 M potassium phosphate containing 2.5 mM $MgCl_2$ (pH 7.0). The sample was digested by the method of Stickland (20), and protein was determined by the micro-biuret method of Goa (7). Bovine serum albumin was used as a standard.

Plate counts and purity of culture checks. Cultures were diluted in 0.85% NaCl and plated onto the hydrogen uptake medium of Maier et al. (12). Counts were made after 5 days of incubation at 28°C.

The following media, used to check for possible contamination of autotrophic cultures, contained these components per liter of medium: yeast extract-mannitol agar (K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; yeast extract, 0.4 g; mannitol, 10 g); yeast extract-tryptone agar (yeast extract, 5 g; tryptone, 8 g; NaCl, 5 g); nutrient agar (beef extract, 3 g; peptone, 5 g). All of these contained 1.5% agar (Difco).

RESULTS

Culture purity. Care was taken during all growth experiments to ensure the absence of bacteriological contamination of cultures. *R. japonicum* SR is resistant to 100 μg of kanamycin per ml and 250 μg of streptomycin per ml. Cells of all autotrophic cultures were plated periodically onto hydrogen uptake medium containing the two antibiotics. Platings were also done on nutrient agar, yeast extract-mannitol agar, and yeast extract-tryptone agar to check for growth of contaminants. Typical cell morphology of *R. japonicum* SR was observed microscopically throughout the experiments, and a single colony type with both antibiotic resistances was always recovered. As would be expected for pure cultures of *R. japonicum*, no growth occurred on nutrient agar or yeast extract-tryptone agar.

Effect of atmospheric composition. To obtain repeatable growth conditions starting with a low-density inoculum, typical experiments were run with an atmosphere of 1% O_2, 5% CO_2, 10% H_2, and 84% N_2 in the incubation jars as described in Materials and Methods. Growth characteristics under these conditions are shown in Fig. 1. Cultures that were not supplied with H_2 did not grow autotrophically, nor did cultures that were not supplied with CO_2 (data not shown). If higher initial O_2 tensions or more vigorous aeration were provided, the cells did not grow. Thus O_2 partial pressure in the gas phase was crucial to the viability of low-density cultures of *R. japonicum* SR grown autotrophically. However, increases in O_2 concentration as growth progressed were stimulatory and prevented cessation of growth in these cultures at
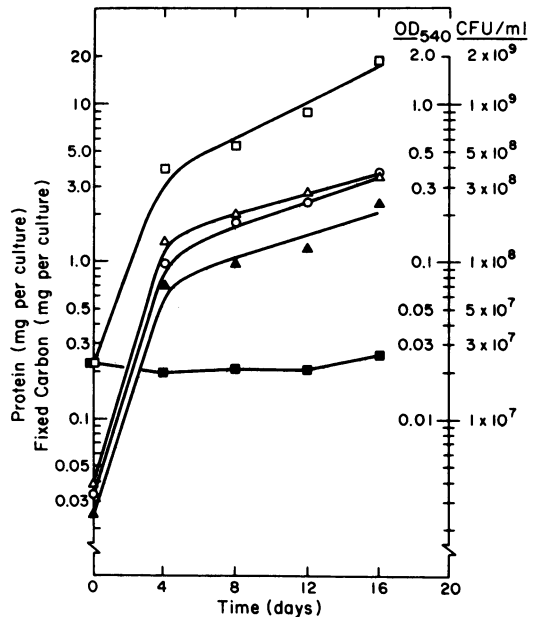

FIG. 1. *Increases in protein (milligrams per culture, △), organic carbon (milligrams per culture, ▲), optical density (at 540 nm, ○), and cell number (colony-forming units per milliliter with H_2, □; without H_2, ■) in autotrophic cultures of R. japonicum SR. A 50-ml sample of medium was inoculated with autotrophically grown cells to contain approximately 2 × 10^7 cells per ml and incubated in an atmosphere containing 10% H_2, 5% CO_2, 1% O_2, and 85% N_2. One set of cultures was incubated without H_2. Medium, culture techniques, and methods for determination of protein and organic carbon are as described in the text. The values presented are the averages of the results of five replicate determinations.*

low cell densities. Under these conditions (1% O_2 maintained), optical densities at 540 nm reached a maximum of about 0.4, after which no further growth was apparent, and $^{14}CO_2$ uptake rates (determined as described in Materials and Methods) dropped precipitiously.

We therefore determined the rate at which oxygen content of the atmosphere could be increased. Figure 2 shows that the programming regime in which O_2 was increased to a maximum of 8% was most effective, resulting in an approximate twofold increase in growth rate over those cultures grown in 1% O_2 atmospheres. Too rapid an increase is detrimental to such cultures even after optical densities of 0.8 have been attained. This contrasts with chemoautotrophic cultures of *Rhodopseudomonas capsulata* (11) or *Aquaspirillum autotrophicum* (2), both of which are able to tolerate 20% or more oxygen at mid-logarithmic growth phase. It is possible that our cultures became limited by factors in the medium at high cell densities.
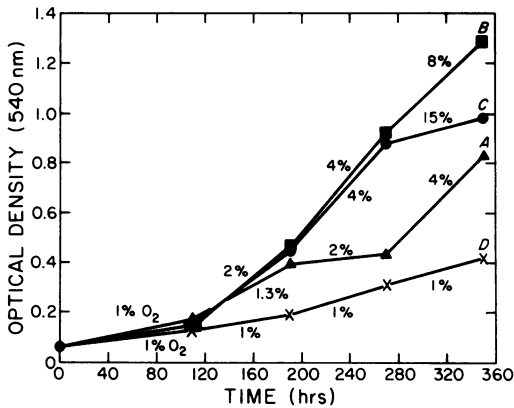
FIG. 2. *Effect of increasing O_2 concentration on autotrophic growth of R. japonicum SR. Four groups of autotrophic cultures of R. japonicum SR were grown in gas mixtures containing 10% H_2, 5% CO_2, and various O_2 concentrations with the balance of the mixture made up by N_2. The percentage of O_2 in the gas phase for each of the four groups was varied at intervals during the growth period as indicated above. Cultures (50 ml) were grown in triplicate in 125-ml bottles with shaking. Samples (1 ml) were taken at 110, 190, 270, and 350 h for optical density measurement, and means of triplicate cultures are reported.*

Effect of nitrogen source. To determine the effect of nitrogen source on autotrophic growth, three 50-ml cultures of mineral salts/vitamins medium were supplemented with 1.35 mg of N added either as NH_4Cl or as KNO_3. Three nitrogen-free control cultures were included. Cultures were inoculated with autotrophically grown cells to contain approximately 10^6 cells per ml, and growth was measured as optical density at 540 nm after 20 days. Gaseous nitrogen did not support autotrophic growth. NH_4Cl-supplemented cultures grew to final densities of about twice those of KNO_3-supplemented cultures.

Contribution of carbon dioxide assimilation to growth. The rate of CO_2 fixation by autotrophically growing cells was measured as described in Materials and Methods. The assay conditions were approximately the same as the conditions under which cultures were grown. Average hourly rates of CO_2 fixation were measured for 5-day-old cultures and 11-day-old cultures, and the amount of carbon expected to be accumulated was calculated based on the arithmetic mean of rates determined over the indicated growth period. These data and the total carbon accumulated at the end of each growth period are shown in Table 1. Total carbon was determined by the chromic acid reduction assay of Seargent and Reiman (18) with modifications described in Materials and Methods. On this basis, 61 and 89% of the carbon accumulated

could be accounted for by the CO_2 flux at 5 days and 11 days, respectively. The fraction accounted for at 5 days is a minimal estimate, since this value is the average of the rate at day 5 and the rate at day 0, which was unmeasurable and taken to be zero. Uptake rates measured after 17 days of growth were very low (data not shown). Although additional carbon had accumulated between 11 and 17 days, the cultures had reached stationary phase, a condition likely due to insufficient O_2 supply; therefore CO_2 fixation was limited.

Autotrophy is a condition of growth in which the majority of the carbon budget is satisified by CO_2 (23). Carbon dioxide is fixed by RuBP carboxylase, an enzyme present in most autotrophically grown cells (1, 16). A specific activity for this enzyme of 6.3 (nmol/min per mg of protein) was reported by Simpson et al. (19) in crude extracts of *R. japonicum* SR cells washed from plates of hydrogen uptake medium. Autotrophically grown cells of three strains of *R. japonicum* (SR, 3I1b6, and USDA 136) were assayed for whole-cell RuBP carboxylase; the activities (Table 2) were of the same order of magnitude as those reported for crude extracts of 12/60X (6), *Pseudomonas oxalaticus* (9), and *A. autotrophicum* (3). Thus, more than adequate RuBP carboxylase specific activity was demonstrated in *R. japonicum*, since the specific activities should be higher in cell-free extracts.

Hydrogenase activity in cells of *R. japonicum* SR grown and derepressed on low-carbon hydrogen uptake medium (19) was about 1/20 of the activities we found in autotrophically grown cells. This comparison is made in Table 2. The activities we obtained are 10-fold those Hanus et al. (8) found with autotrophically grown cells of the same strain, and 27-fold and 16-fold, respectively, the activities reported by Ruiz-Argüeso et al. (17) and McCrae et al. (14) for *R.*

TABLE 1. *Extent of $^{14}CO_2$ fixation by autotrophic cultures of R. japonicum SR[a]*

Growth period (days)	Total carbon accumulated per 50 ml of culture (mg)	Calculated carbon fixed from $^{14}CO_2$ uptake (mg)	Fraction of carbon from CO_2 (%)
0–5	0.18 ± 0.02	0.11 ± 0.03	61
0–11	1.28 ± 0.07	1.14 ± 0.07	89

[a] Cells were cultured for the indicated growth period, and CO_2 uptake rates were determined as described in the text. Total carbon was assayed by the method of Seargent and Reiman (18), with modifications described in the text. The percentage of carbon from CO_2 was then calculated based on the average rates of $^{14}CO_2$ assimilation over the designated growth period. All values represent at least triplicate determination ± standard error of the mean.

TABLE 2. *Activity of RuBP carboxylase and hydrogenase of R. japonicum strains after heterotrophic or autotrophic growth*[a]

Strain	Growth conditions	Hydrogenase sp act (μmol/min per mg of protein)	RuBP carboxylase (nmol/min per mg of protein)
SR	Hydrogen uptake medium	0.058	6.3
SR	Autotrophic medium	1.025	80.0
3I1b6	Autotrophic medium	ND	78.0
136	Autotrophic medium	ND	85.0

[a] Cells were grown for 3 days in air on plates of hydrogen uptake medium (12) and for 3 additional days under an atmosphere of 84% N_2, 10% H_2, 5% CO_2, and 1% O_2. Cells from these plates, suspended in 0.05 M potassium phosphate (pH 7.0) with 2.5 mM $MgCl_2$, were assayed amperometrically for hydrogenase as described by Maier et al. (13). Data shown for growth on H_2 uptake medium are taken from Simpson et al. (19). After 16 days of autotrophic growth, as described in the text, whole-cell hydrogenase was assayed amperometrically (value is average of five replicate cultures). Whole-cell RuBP carboxylase was assayed by the method of Tabita et al. (21). ND, Not done.

japonicum bacteroid preparations. Hydrogen uptake activities of our cultures were 47-fold those reported by Maier et al. (12) for free-living bacteria grown and derepressed under the best conditions they were able to devise.

The identity of the RuBP carboxylase reaction product as 3-phosphoglycerate, and the stoichiometry of about 2 mol of product per mol of CO_2 fixed, were established. The data and experimental details are given in Table 3. No biological substrate is known which can react in place of 3-phosphoglycerate in the coupled enzyme assay that was used for the determination (5).

DISCUSSION

Rhizobia have been known for several years to require CO_2 for growth (10), and more recent investigations (4, 15a, 19) have shown the presence of propionyl coenzyme A carboxylase in both free-living and bacteroid forms of R. japonicum. Moreover, the discovery by Simpson et al. (19) that RuBP-dependent fixation of CO_2 occurs in cell-free extracts of R. japonicum has evoked considerations of a possible close phylogenetic relationship of Rhizobium spp. to hydrogen bacteria, and certainly forces reconsideration of the metabolic flexibility and ecological niche of the organism. This investigation has centered on further characterization of autotrophic growth of R. japonicum. More optimal growth conditions have been developed, under which concomitant increases in the specific ac-

tivities of two enzymes necessary for chemoautotrophic growth, hydrogenase and RuBP carboxylase, have been observed.

We found the specific activity of uptake hydrogenase during autotrophic growth of R. japonicum to be severalfold higher than activities for the same organism grown under any condition heretofore reported. The H_2 uptake medium of Maier et al. (12) is a low-carbon medium, but it is conceivable that some repression of hydrogenase is effected by this carbon; we found 47-fold the specific activity that they report for free-living cells grown on that medium. Simpson et al. (19) report that although propionyl coenzyme A carboxylase is expressed by bacteroids, RuBP carboxylase is not detectable. If RuBP carboxylase and hydrogenase are coordinately regulated as they claim, and the carboxylase is repressed in the bacteroid, it seems reasonable that hydrogenase might be at least partially repressed. Thus organic carbon available from the nodule cytosol might account for the lower activities that McCrae et al. (14) and Ruiz-Argüeso et al. (17) report for bacteroid preparations. R. japonicum grown autotrophically by Hanus et al. (8) was shown to have 1/10 the hydrogenase activity of our cultures, but their cultures may have been senescent when the enzyme was assayed. Thus it seems that uptake hydrogenase activities of autotrophically grown R. japonicum are manyfold those obtainable from cells grown under other conditions. This phenomenon may prove useful in study or purification of hydrogenase from cells capable of such growth. During autotrophic growth, cells are forced to depend on H_2 uptake for energy to drive carbon fixation. It is advantageous that such cells should have the most highly induced and efficient uptake hydrogenase.

Simpson et al. (19) report RuBP carboxylase activity of R. japonicum derepressed for uptake

TABLE 3. *Stoichiometry of R. japonicum RuBP carboxylase*[a]

Assay conditions	CO_2 fixed (nmol)	3-Phosphoglycerate produced (nmol)	3-Phosphoglycerate/CO_2
+RuBP	160.0	301.0	1.88
−RuBP	<2.0	<1.0	—

[a] Crude cell-free extracts with a specific activity of 83 nmol of CO_2 fixed per min per mg of protein were used. RuBP carboxylase reaction mixtures of double the usual volume (0.5 ml) were incubated for 10 min, after which 0.2 ml was removed for determination of the amount of CO_2 fixed as described in the text. The remaining 0.3 ml of the reaction mixture was placed in boiling water for 5 min and then stored at −20°C until 3-phosphoglycerate estimations could be made (5).

hydrogenase to be 1/12 of the activities we demonstrate here in autotrophically grown cells. Again, since the cells used in their experiments were washed from medium containing some carbon, it may be assumed that the carbon-fixing enzyme of autotrophic growth may be partly repressed.

Autotrophic growth of *A. autotrophicum* was shown to be limited by excessive partial pressure of O_2 during early growth, but as growth proceeded O_2 supply had to be adapted to the increasing demand (2). Madigan and Gest (11) reported that hydrogen-supported chemoautotrophic growth of *R. capsulata* was inhibited by O_2 concentrations of 20% during initial growth, but that an increase from 10% to 20% O_2 during the mid-logarithmic growth phase increased growth rates and prevented the cultures from entering the stationary phase prematurely. Similarly, we found that at low cell densities (2 × 10^7 cells per ml or less), oxygen had to be limited to 1% of the atmosphere to avoid killing the cultures. However, greatly enhanced growth was obtainable by programming an increase in O_2 tension.

Finally, conservative estimates of the rate of CO_2 assimilation during the logarithmic phase of growth show that these rates are adequate to account for 89% of the carbon that has accumulated. Furthermore, RuBP carboxylase activities in intact cells of three strains of autotrophically growing *R. japonicum* were sufficient to account for over four times the total carbon accumulated at any point assayed during 16 days of growth, and the rates of $^{14}CO_2$ uptake (data not shown) measured at any of several points during the growth of *R. japonicum* SR could be accounted for by the activity of RuBP-carboxylase alone. 3-Phosphoglycerate was shown to be the product of RuBP-dependent fixation of carbon dioxide with a molar ratio of about 2 mol of phosphoglycerate to 1 mol of CO_2. This is the stoichiometry expected of RuBP carboxylase-catalyzed carbon fixation (16). These findings unequivocally establish the enzyme's role in catalysis of the cardinal route of carbon assimilation for autotrophically growing *R. japonicum* SR.

Thus we have quantitated the extent to which the CO_2 fixation by autotrophically growing *R. japonicum* SR may contribute to their carbon budget. Given the proper conditions, virtually all carbon may enter the cell in this manner. With improved growth media and culture conditions, we clearly show that vigorous growth may be expected. Hanus et al. (8), while discussing their original observation of the phenomenon of rhizobial autotrophy, point out the potential

survival value in the competitive soil environment that autotrophy may afford. Since only those rhizobia possessing the uptake hydrogenase capability have been demonstrated to grow autotrophically, it will be interesting to investigate whether those strains that are wildtype and uptake-hydrogenase negative possess the ancillary physiological machinery necessary for autotrophic growth. This is being approached in our laboratory by genetic transfer of hydrogenase into such strains. The fruits of such manipulation should include a better understanding of the possible close evolutionary relationship between the rhizobia and the hydrogen bacteria. Additionally, a more satisfying answer may be provided to the question of whether autotrophy and uptake-hydrogenase function are vestigal mechanisms of a common ancestry with the hydrogen bacteria, or whether these capabilities continue to afford significant adaptive advantage for rhizobia in the wild.

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