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Regulation of Hydrogenase in *Rhizobium japonicum*: Analysis of Mutants Altered in Regulation by Carbon Substrates and Oxygen†

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The synthesis of the H_2 uptake system in free-living *Rhizobium japonicum* SR is repressed both by oxygen and by carbon substrates. Mutants selected for the ability to express hydrogenase in 10.0% partial pressure O_2 were also less sensitive than the wild type to repression by carbon substrates such as arabinose, glycerol, gluconate, and succinate. The H_2 uptake system in another class of mutants, previously shown to be hypersensitive to repression by O_2, is also more sensitive to repression by carbon substrates. The oxygen- and carbon-insensitive mutants express the hydrogen uptake system during heterotrophic growth in the absence of hydrogen and thus can be considered constitutive (Hup^c). The amount of cytochromes in the Hup^c mutants is similar to that in the wild-type strain; however, the Hup^c mutants contain greater methylene blue-dependent and O_2-dependent hydrogenase activity, both as free-living cells and as bacteroids. Two-dimensional polyacrylamide gel electrophoresis revealed that during heterotrophic growth the Hup^c mutant strain SR470 synthesized at least six peptides not found in the wild-type strain. The concentrations of cyclic AMP and guanosine tetraphosphate were similar in strain SR and the Hup^c mutants during heterotrophic growth.

Some nitrogen-fixing organisms are capable of oxidizing the H_2 evolved by nitrogenase via a hydrogen uptake system. The H_2 uptake system of *Rhizobium japonicum* increases the efficiency of nitrogen fixation in soybean root nodules (11). Hydrogen uptake activity can also be expressed in free-living *R. japonicum*, enabling cells to grow chemoautotrophically with H_2 as a source of energy (11, 13, 16).

A variety of environmental factors affect synthesis of the *R. japonicum* H_2 uptake system. In wild-type free-living cells hydrogenase is not expressed in the presence of high concentrations of carbon substrates or oxygen partial pressures greater than 4.0% (13, 19–21, 28). In addition, hydrogen and carbon dioxide stimulate synthesis of hydrogenase by free-living cells (13, 20, 28). Nothing is known of the factors that regulate the synthesis of hydrogenase in bacteroids. Since an environment low in oxygen is required for hydrogenase expression in free-living cells, it is possible that the microaerophilic environment of the root nodule may be important for the enzyme synthesis in bacteroids. However, it is interesting that bacteroids are supplied with adequate carbon substrates by the plant (26), whereas free-living cells must be incubated in a medium lacking carbon for expression of hydrogenase (19, 20, 28).

We previously described regulatory mutants in which expression of hydrogenase was hypersensitive to repression by O_2 (21) and also mutants in which hydrogenase was not sensitive to repression by O_2 (24). In this report, we present the results of additional studies on both types of mutants. We have found that mutants altered in O_2-mediated regulation of hydrogenase are also altered in the normal carbon-mediated regulation of the enzyme. These studies suggest that a common element is involved in the regulation of the uptake hydrogenase by oxygen and by carbon substrates.

MATERIALS AND METHODS

Chemicals. L-Arabinose, sucrose, sodium gluconate, sodium succinate, guanosine tetraphosphate, and methylene blue were obtained from Sigma Chemical Co., St. Louis, Mo. High-specific-activity (50 mCi/mg-atom of carbon) uniformly ^{14}C-labeled amino acids were purchased from Amersham Corp., Arlington Heights, Ill. Potassium phosphate and other mineral salts for the routine culture of bacteria were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. H_2, N_2, O_2 and gas mixtures were supplied by Arundel Sales and Service Co., Baltimore, Md.

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TABLE 1. Bacterial strains

Phenotype	Strain	Type of mutation	Reference
Insensitive to O_2 repression[a]	SR470	Spontaneous	25
	SR471, SR472	EMS induced[b]	This work
	SR473, SR474	EMS induced	25, this work
	SR475, SR476	EMS induced	This work
	SR477, SR478	Spontaneous	25, this work
	SR479, SR480	Spontaneous	This work
	SR481, SR482	Spontaneous	This work
Hypersensitive to O_2 repression	SR174, SR178, SR186	EMS induced	22

[a] Each mutant or pair of mutants listed is from an independent mutant selection and screening. The mutant selection screening procedure is described in reference 24.

[b] The culture was treated with ethyl methane sulfonate to kill approximately 90% of the cells as described previously (18).

Strains. *R. japonicum* SR has been described previously (18, 23). Spontaneous and ethyl methane sulfonate-induced mutants derived from SR are listed in Table 1. Seven independent mutant selections and screenings were used to obtain the 13 O_2-insensitive mutants (Table 1). The O_2-hypersensitive mutants were described previously (21).

Culture conditions. *R. japonicum* was cultivated heterotrophically in liquid-modified Bergersen medium as described by Bishop et al. (4) or on solidified hydrogen uptake medium as described by Maier et al. (19). Heterotrophically grown cultures were derepressed for hydrogen uptake activity by suspending cells in 0.05 M potassium phosphate buffer (pH 7.0) containing 2.5 mM $MgCl_2$ to a concentration of 5×10^8 cells per ml (corresponding to approximately 85 μg of cell protein per ml) as previously described (18). Small volumes (10 ml) of culture were derepressed in 250-ml flint glass bottles; larger volumes (up to 1 liter) were derepressed in 4-liter Erlenmeyer flasks. The vessels were sealed and flushed with N_2, followed by flushing with a mixture containing 85% N_2, 10% H_2, and 5% CO_2. The oxygen content of the gas phase was adjusted to the indicated partial pressure by the injection of 100% oxygen. The derepressing cultures were incubated at 30°C on a gyratory shaker (140 cycles per min) for 20 to 24 h.

Growth of soybean plants and harvest of bacteroids. Procedures for the cultivation of soybean plants (cultivar Essex) and harvesting of bacteroids have been described previously (24).

Isolation of membrane fraction and measurement of cytochrome concentrations. Cells were broken in a French pressure cell, and the membrane fraction was isolated as described previously (22). Total cytochrome concentrations were calculated from dithionite-reduced minus air-oxidized spectra recorded on a Perkin-Elmer model 557 spectrophotometer in the dual beam mode; the extinction coefficients for quantitating cytochromes *c*, *b*, and aa_3 were as described by Appleby (1).

H_2 and O_2 uptake assays. The rate of H_2 uptake was measured amperometrically (12, 29, 30). The assay mixture contained the following (in 4.8 ml): 240 μmol of potassium phosphate (pH 7.0), 12 μmol of $MgCl_2$, and 37.7 nmol of H_2. For the measurement of O_2-dependent H_2 uptake by whole cells, the assay mixture contained 2.5×10^9 cells and was air saturated. The optical density at 540 nm was measured, and the cell number was estimated from a standard curve of optical density at 540 nm versus viable cell number. For the measurement of methylene blue-dependent H_2 uptake in membranes, all assay components were sparged with argon; the mixture also contained 0.5 μmol of dithionite, 500 nmol of methylene blue, and 60 to 125 μg of protein from the membrane preparation. Oxygen uptake was measured amperometrically, essentially as described by Hanus et al. (12). Protein concentration was estimated by the method of Bradford (7).

Two-dimensional polyacrylamide gel electrophoresis. *R. japonicum* was cultivated in 25 ml of Bergersen medium to a cell density of approximately 4.0×10^8 cell per ml. O_2-dependent H_2 uptake was measured before the addition of radiolabeled amino acids and was 119 nmol of H_2 per h per 10^8 cells for SR470. The wild type had no detectable activity. Radiolabeled amino acid mixture (2.5 μCi of high-specific-activity uniformly, ^{14}C-labeled amino acids) was added, and the cells were allowed to grow for an additional 4 h. The cultures were then placed on ice, centrifuged, and prepared for electrophoresis by the method of Roberts et al. (27), followed by two-dimensional electrophoresis essentially as described by O'Farrell (25). The first-dimension gel was loaded with 10^5 cpm. The acrylamide concentration in the second dimension was 12%. After fixation in a solution containing 10% acetic acid and 10% methanol for 30 min, the gels were prepared for fluorography by the method of Lasky and Mills (14), dried, and clamped against Kodak XAR-5 film for 1 week at −70°C.

Determination of concentrations of cAMP and ppGpp. Cyclic AMP (cAMP) was determined by radioimmunoassay as described by Lim and Shanmugam (17) with a cAMP assay kit (New England Nuclear Corp., Boston, Mass.). Guanosine tetraphosphate (ppGpp) was determined by thin-layer chromatography (8). A 10- to 20-μl sample of a ^{32}P-labeled extract of each strain was applied to polyethyleneimine-cellulose thin-layer sheets (Brinkmann Instruments Inc., Westbury, N.Y.) and chromatographed in 1.5 M KH_2PO_4 (pH 3.4). Purified ppGpp was applied in the same lanes to facilitate identification of labeled nucleotides. After autoradiography the ppGpp spots were cut out and counted in a liquid scintillation counter.

RESULTS

Expression of hydrogen uptake activity by O_2-insensitive strains in the presence of carbon sub-

strates. The O_2-insensitive strains we studied in the most detail were SR470, SR473, and SR478. Each of these strains came from an independent mutant selection and screening; two of these strains are spontaneous mutants, whereas one (SR473) was ethyl methane sulfonate induced (Table 1). We found that the respiration rates of strains SR, SR470, SR473, and SR478 in heterotrophic growth medium were very similar (35.0 to 44.0 nmol/10^8 cells per h). Therefore the mutants are not defective in respiratory activity. To characterize the mutant strains further, we compared the effect of carbon substrates on derepression of hydrogen-oxidizing activity in the mutant and wild-type strains. In the presence of 50 mM arabinose, 90% of the hydrogen-oxidizing activity in strain SR was repressed. However, in strains SR470, SR473, and SR478 the addition of 50 mM arabinose reduced the expression of hydrogenase activity by only 30 to 40% (Table 2). In similar experiments, 10 mM glycerol or 50 mM gluconate also repressed hydrogen uptake activity to a much greater extent in strain SR than in the mutants (data not shown).

The addition of 1 mM succinate reduced expression of hydrogenase by 33% in strain SR and by 24% in strain SR470; with 10 mM succinate, over 90% of the hydrogenase activity of strain SR was repressed, whereas strain SR470 expressed approximately 50% of maximum activity (Table 3). Similar results were obtained with the other mutants, SR473 and SR478. These data demonstrate that the hydrogen uptake system is less sensitive to repression by carbon substrates in the mutant strains.

In the above experiments the carbon substrate was added to resting cells (i.e., cells incubated

TABLE 2. Effect of arabinose on expression of hydrogenase in strains SR, SR470, SR473, and SR478[a]

Strain	H_2 uptake	
	No addition	50 mM arabinose
SR	161	12
SR470	223	92
SR473	245	72
SR478	221	67

[a] Strains were derepressed for 22 h in an atmosphere of 10% H_2, 5% CO_2, 1% O_2, and 84% N_2. The cultures were shaken at 100 cycles per min during derepression. Arabinose was added to the designated cultures at the start of derepression. After derepression cells were assayed for H_2 uptake amperometrically with oxygen as the terminal electron acceptor as described in the text. Each value is the mean of the activities from two individual derepressed cultures, and activities are expressed in nanomoles per hour per 10^8 cells.

TABLE 3. Effect of succinate on expression of hydrogen uptake activity in strains SR and SR470[a]

Strain	% of activity expressed relative to control			
	No. addition	Succinate added		
		1.0 mM	5.0 mM	10.0 mM
SR	100[b]	67	37	7
SR470	100[c]	76	80	48

[a] Conditions were as described in Table 1, except that cultures were derepressed for 24 h, the shaking speed was 140 cycles per min, and the carbon substrate added was succinate.

[b] Control value was 68 nmol/10^8 cells per h.

[c] Control value was 160 nmol/10^8 cells per h.

without a nitrogen source) in the presence of 1% O_2. Because the mutants do not require low O_2 concentrations for expression of hydrogen-oxidizing activity (24), it seemed plausible that these strains might express hydrogenase during aerobic growth on organic carbon sources (heterotrophically). We therefore measured hydrogen uptake activity in strains SR, SR470, SR473, and SR478 cultivated in Bergersen medium aerobically. No detectable hydrogen uptake activity (<1.0 nmol/h per 10^8 cells) was produced by strain SR. However, the strains SR470, SR473, and SR478 oxidized 26, 50, and 39 nmol of H_2 per h per 10^8 cells, respectively. Thus these strains do not require the addition of hydrogen during incubation for the expression of hydrogen-oxidizing activity, and they can be considered constitutive mutants (Hup^c). In addition to strains SR470, SR473, and SR478 we have isolated 10 additional Hup^c mutants in seven different mutant selection experiments (Table 1): all of them are less sensitive to both oxygen and carbon repression than the parent strain SR.

Effect of carbon substrates on synthesis of

TABLE 4. Effect of the addition of carbon substrates on expression of the hydrogen uptake system in O_2-hypersensitive mutants

Strain	Expt 1		Expt 2	
	No addition	2.0 mM arabinose	No addition	2.5 mM gluconate
SR	68.0	63.3	53.2	43.8
SR174	7.8	1.9	7.0	1.7
SR178	6.2	1.2	15.3	2.7
SR186	10.9	2.5	15.1	5.5

[a] Strains were derepressed for 20 h in an atmosphere composed of 10% H_2, 5% CO_2, 0.4% O_2, and the balance N_2. The carbon substrate was added to the designated cultures at the start of derepression. After derepression, cells were assayed for H_2 uptake amperometrically with oxygen as the terminal electron acceptor as described in the text. Each value is the mean of the activities from two individual derepressed cultures.

TABLE 5. Cytochrome content and methylene blue-dependent hydrogen uptake in strains SR, SR470, SR473, and SR478[a]

Strain	Free-living cells				Bacteroids		
	MB-dependent H_2 uptake[b]	Cytochromes[c]			MB-dependent H_2 uptake[b]	Cytochromes[c]	
		c	*b*	*aa*$_3$		*c*	*b*
SR	ND[d]	0.12	0.14	0.06	4.8	0.31	0.42
SR470	1.3	0.13	0.15	0.07	23.3	0.36	0.51
SR473	2.7	0.09	0.16	0.07	27.5	0.41	0.46
SR478	1.1	0.11	0.14	0.07	29.8	0.41	0.56

[a] Free-living cells were cultivated in Bergersen medium to approximately 5.0×10^8 cells per ml. The methods for harvesting bacteroids, isolating membranes, assaying methylene blue-dependent H_2 uptake, and determining cytochrome concentrations from dithionite-reduced minus air-oxidized spectra are described in the text.

[b] Activities are expressed in micromoles per hour per milligram protein and are averages of duplicate assays. MB, Methylene blue.

[c] Cytochrome concentrations are expressed as nanomoles of heme per milligram of protein.

[d] ND, No activity detected.

hydrogenase in O_2-sensitive mutants. The effect of carbon substrates on expression of hydrogenase in O_2-sensitive mutants was compared with that in the wild type. Derepression of the hydrogen-oxidizing system in wild-type strain SR was relatively unchanged by the addition of 2 mM arabinose; however, the same concentration of arabinose reduced the expression of hydrogenase by approximately 75% in the oxygen-hypersensitive mutants SR174, SR178, and SR186 (Table 4). In the wild-type strain, the expression of hydrogen uptake activity was reduced by less than 20% in the presence of 2.5 mM sodium gluconate; however, in the mutant strains hydrogenase activity was decreased by 65 to 80% under the same conditions.

Cytochrome content of Hup^c mutants. The total cytochrome content was determined from reduced minus oxidized spectra of membranes prepared from cells cultivated in modified Bergersen medium (Table 5). The oxygen-insensitive mutants did not produce significantly higher levels of cytochrome *c*, *b*, or *aa*$_3$ than did the wild-type strain. However, the mutants synthesized the hydrogen-activating enzyme activity (Table 5), as determined from rates of methylene blue-dependent H_2 uptake (2), in contrast to that of the wild type when grown heterotrophically.

As reported previously (24), bacteroids of the Hup^c mutants contain four- to sixfold more hydrogen-activating enzyme than do bacteroids of the wild-type strain (Table 5). On the other hand, the concentrations of cytochromes *c* and *b* (bacteroids of strain SR do not have cytochrome *aa*$_3$) were similar in bacteroids of the wild-type and mutant strains (Table 5). Thus, although the mutation responsible for constitutive expression of hydrogen-oxidizing activity affects the hydrogen-activating enzyme, no significant effect on cytochromes was detected.

Two-dimensional gel electrophoresis of Hup^c and wild-type strains. Information on polypeptides synthesized by *R. japonicum* was obtained by examining extracts of strains SR and SR470 by two-dimensional polyacrylamide gel electrophoresis. Extracts were prepared from cells growing heterotrophically. At least six polypeptides were synthesized by strain SR470, but not by strain SR (Fig. 1). We did not detect any proteins present in the wild-type strain that were not present in the mutant. Other than the six peptides designated in Fig. 1, the two-dimensional gel protein pattern of the Hup^c mutant was like that of the wild-type strain.

Relative concentrations of cAMP and ppGpp in Hup^c and wild-type cells. Lim and Shanmugam reported a 10-fold increase in intracellular cAMP levels associated with the induction of hydrogen uptake activity in *R. japonicum* (17). To determine whether constitutive hydrogenase synthesis in the mutant strains results from overproduction of cAMP, we measured cAMP concentrations in the Hup^c and wild-type strains cultivated heterotrophically. The mutants contained only 25 to 50% higher concentrations of cAMP than did strain SR (Table 6). This increase is much smaller than the increase associated with hydrogenase expression in wild-type cells reported by Lim and Shanmugam (17) ppGpp has also been implicated as a regulator of catabolic enzymes (5), and the intracellular concentration of ppGpp is increased in *Rhizobium meliloti* during carbon starvation (3). However, there was no significant difference in the amount of ppGpp observed in heterotrophically grown SR and Hup^c mutants (Table 6).

DISCUSSION

Hup^c mutants were selected on the basis of their ability to utilize hydrogen as an energy source in the presence of high concentrations of oxygen (24). The lesion in these strains appar-

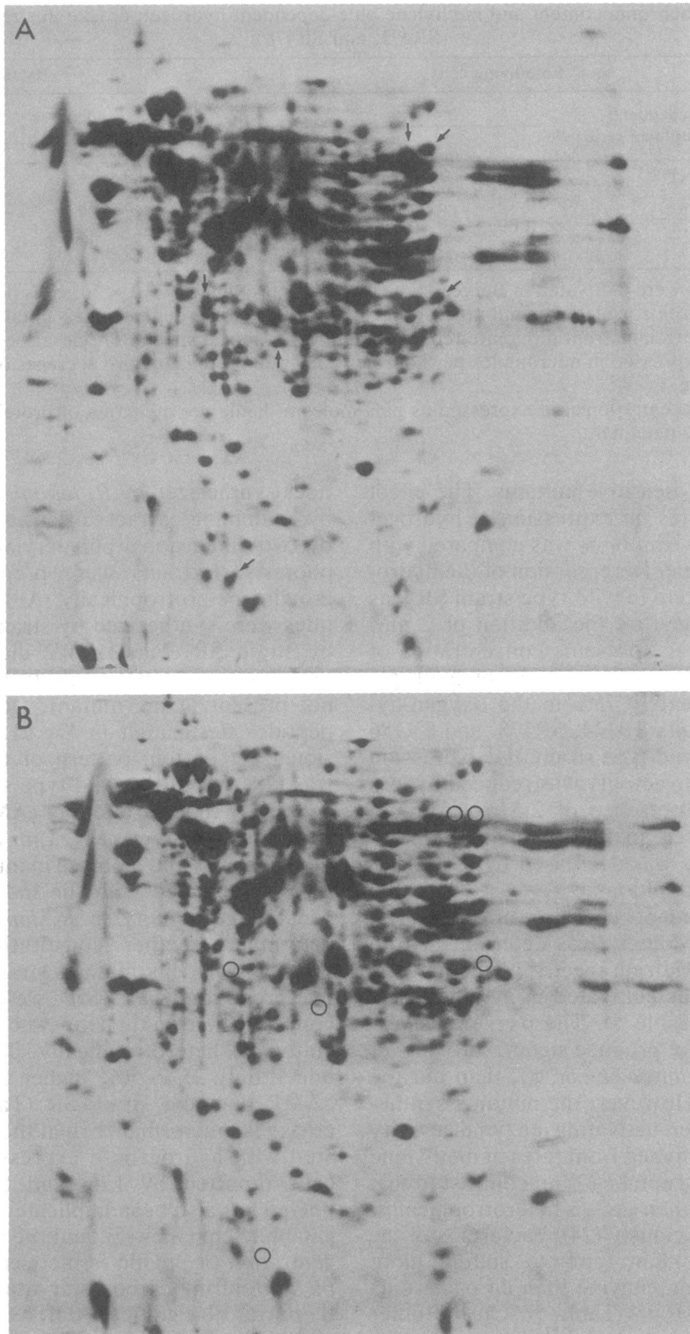

FIG. 1. Two-dimensional gels of extracts of *R. japonicum* were prepared as described in the text. (A) Strain SR470; arrows designate peptides not present in extract of strain SR. (B) Strain SR; areas that do not contain peptides, but that correspond to peptides produced by strain SR470, are marked by circles. Similar patterns were obtained in two additional experiments.

ently affects the first step in hydrogen oxidation, the activation of hydrogen, since we observed no difference in the cytochrome content of the mutant and wild-type strains.

The constitutive mutants are unlike the parent strain in that they synthesize the hydrogen uptake system in the absence of H_2 and in the presence of organic carbon or high concentra-

TABLE 6. Concentrations of cAMP and ppGpp in strains SR, SR470, SR473, SR478

Strain	pmol of cAMP/10^8 cells[a]	ppGpp (cpm)[b]
SR	0.13 ± 0.02	5,685
SR470	0.16 ± 0.01	6,323
SR473	0.18 ± 0.01	7,989
SR478	0.19 ± 0.01	5,841

[a] Strains were cultivated in Bergersen medium to 5.0×10^8 cells per ml, and cAMP was determined as described in the text. Values are means ± standard deviations of five replicate samples.

[b] Strains were cultivated in phosphate-free *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid–morpholineethanesulfonic acid–arabinose medium (9) to 5.0×10^8 cells per ml. Samples (100 μl) of the cultures were labeled for 30 min with 500 μCi of carrier-free ^{32}P (Amersham) per ml. Incorporation was stopped, and nucleotides were extracted by the addition of 100 μl of ice-cold 2.0 M formic acid. After a 30-min incubation at 0°C the extract was clarified by centrifugation for 1 min at 13,000 × *g*, and samples (10 to 20 μl) were applied to chromatograms and fractionated as described in the text.

tions of oxygen. Increased synthesis of the hydrogen uptake system in the presence of carbon substrates is consistent with the observation that the Hup^c mutants contain more hydrogen uptake activity than does the parent strain as bacteroids in soybean nodules (24), where carbon sources are plentiful (26). In addition, mutants that show an increased sensitivity to repression of hydrogenase by oxygen are also more sensitive to repression by carbon substrates. Based on these findings, we suggest that there is a common regulatory element involved in the control of hydrogenase synthesis by oxygen and carbon. Descriptions of carbon and oxygen repression of hydrogenase expression in H_2-oxidizing bacteria are common (6). However, we are not aware of any other mutants in which the regulation of hydrogenase by both carbon and oxygen is affected.

Conceivably, the hydrogen uptake system is subject to catabolite repression-like regulation, a phenomenon that has been linked to regulation by carbon substrates and respiratory electron acceptors (5, 10, 15). We have observed only minor differences in the concentrations of cAMP in the mutant and wild-type strains; however, it is important to note that variation in cAMP concentration does not completely account for catabolite repression in other organisms (5, 15).

Analysis of cell extracts by two-dimensional gel electrophoresis has revealed that the Hup^c mutant strain SR470 synthesizes at least six polypeptides that are not produced by wild-type cells growing heterotrophically. Presumably one of these peptides is the hydrogen-activating enzyme; other peptides may be involved in regulation of hydrogenase or may be coregulated with hydrogenase. This observation is consistent with a hypothesis that the gene affected in the Hup^c mutant controls the synthesis of several enzymes.

The isolation of viable mutant strains capable of autotrophic growth in the presence of high partial pressures of oxygen raises the question of why the hydrogen uptake system in the wild-type strain is normally repressed by oxygen. Several species of H_2-oxidizing bacteria grow autotrophically in high concentrations of O_2 (6). Perhaps the explanation involves the fact that in *R. japonicum* hydrogenase is normally expressed in bacteroids, which exist in a low-O_2 environment.

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