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Regulation of Hydrogenase in *Rhizobium japonicum*

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Factors that regulate the expression of an H_2 uptake system in free-living cultures of *Rhizobium japonicum* have been investigated. Rapid rates of H_2 uptake by *R. japonicum* were obtained by incubation of cell suspensions in a Mg-phosphate buffer under a gas phase of 86.7% N_2, 8.3% H_2, 4.2% CO_2, and 0.8% O_2. Cultures incubated under conditions comparable with those above, with the exception that Ar replaced H_2, showed no hydrogenase activity. When H_2 was removed after initiation of hydrogenase derepression, further increase in hydrogenase activity ceased. Nitrogenase activity was not essential for expression of hydrogenase activity. All usable carbon substrates tested repressed hydrogenase formation, but none of them inhibited hydrogenase activity. No effect on hydrogenase formation was observed from the addition of KNO_3 or NH_4Cl at 10 mM. Oxygen repressed hydrogenase formation, but did not inhibit activity of the enzyme in whole cells. The addition of rifampin or chloramphenicol to derepressed cultures resulted in inhibition of enzyme formation similar to that observed by O_2 repression. The removal of CO_2 during derepression caused a decrease in the rate of hydrogenase formation. No direct effect of CO_2 on hydrogenase activity was observed.

The *Rhizobium*-legume symbiosis utilizes energy produced from photosynthate to fix N_2, allowing the plant to grow without added nitrogenous compounds. The ATP-dependent evolution of H_2, by the nitrogenase reaction appears to be a significant means whereby energy is lost during the N_2-fixing process (8, 18). The evolution of H_2 during N_2 fixation apparently is an inherent characteristic of the nitrogenase reaction (14). Some strains of *Rhizobium*, however, produce nodules containing an uptake hydrogenase which is involved in a recycling process that utilizes the H_2 produced from the nitrogenase reaction (5, 18). The oxidation of H_2 by nodule bacteroids can protect nitrogenase from O_2 inactivation, provide energy for ATP synthesis (6, 7), and conserve carbon substrates within the bacteroids (11). These beneficial effects may result in significant increases in plant dry matter (19). Little is known about the regulation of hydrogenase in *Rhizobium*. The hydrogenase system in *Rhizobium* is complex, catalyzing the activation and oxidation of H_2 through a series of unidentified electron transport components yielding water as the final product. The term "hydrogenase" in the paper therefore includes the entire system involved in H_2 uptake and oxidation.

Hydrogenase activity can be readily demonstrated in the H_2-uptake positive strains of *R. japonicum* bacteroids isolated from legume nodules. Recently, the conditions required for expression of hydrogenase in free-living *R. japonicum* were described (12). Low concentrations of carbon substrates and a low partial pressure of O_2 (1% in the gas phase) were required for high H_2 uptake activities. These results suggest that carbon substrates and O_2 may be involved in the regulation of hydrogenase synthesis. Hydrogenase activity in *R. japonicum* cultures was dependent upon a preincubation period of the cells in the presence of H_2. Preincubation with H_2, however, was not required under conditions where nitrogenase activity was expressed. From these results it appears that H_2 participates in the control of hydrogenase synthesis. Nitrogenase activity in *Rhizobium* cells, however, is not necessary for hydrogenase formation and expression. Since H_2 apparently was necessary for induction of hydrogenase, an investigation of the factors that regulate hydrogenase formation in free-living cultures of *R. japonicum* seemed necessary.

MATERIALS AND METHODS

Chemicals. Sodium gluconate, L-arabinose, sodium glutamate, rifampin, and chloramphenicol were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium citrate, glycerol, sorbitol, and potassium nitrate were obtained from Mallinckrodt, Inc., St. Louis, Mo. Sodium succinate and sucrose were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J.

Hydrogenase derepression. *R. japonicum* strain

USDA 122 (DES) used in all these experiments is a small colony derivative of USDA 122 which was isolated by David Emerich of this laboratory using a detergent dilution procedure (9). Strain 122 (DES) was inoculated onto agar slants of a medium specifically designed for expression of hydrogenase activity (12). After 5 days of growth at 26°C in air, the cells were washed off the slants with 5 ml of Mg-phosphate buffer (0.05 M potassium phosphate-2.5 mM $MgCl_2$ adjusted to pH 7.0). The cells were suspended by agitation, and diluted to an optical density at 540 nm of 0.5 with Mg-phosphate buffer. This corresponds to approximately 4×10^8 viable cells per ml. A 6-ml portion of the cell suspension was added to a 245-ml prescription bottle, and the bottle was sealed with a serum stopper and then flushed with N_2 until no remaining O_2 could be detected by gas chromatography. The 6-ml cell suspension resulted in about 3-cm depth of liquid in the bottle, allowing rapid gas equilibration into the culture. Sufficient H_2, CO_2, and O_2 were added to each bottle to obtain an atmosphere composed of 86.7% N_2, 8.3% H_2, 4.2% CO_2, and 0.8% O_2, and then bottles were incubated at 26°C. The conditions for testing repression by organic compounds are described in footnote *a* of Table 2.

H_2 uptake assays. Rates of H_2 uptake were measured amperometrically by methods described previously (18, 20). The 6-ml portion of the suspension of hydrogenase-derepressed cells from a 245-ml bottle was pipetted into a 21-ml serum vial which was flushed with N_2. The cells were transferred by syringe to the 5.8-ml cuvette of the amperometric device, and 62 nmol of O_2 and 37.7 nmol of H_2 (as saturated solutions of these gases) were added to the cuvette to initiate the assay. These concentrations of gases were sufficient for saturation of the H_2 uptake reaction. After the H_2 uptake measurement, the optical density of the suspension at 540 nm was measured and the cell number was determined from standard curves based upon plate counts.

RESULTS

Induction by H_2. An investigation of the regulation of hydrogenase in *R. japonicum* required relatively high H_2 uptake rates of cells from liquid culture. In our previous investigations of hydrogenase expression in free-living cultures of *R. japonicum*, cells were cultured on agar slants and assayed for activity without removal from the medium (12). Our preliminary attempts to obtain hydrogenase activity in free-living liquid cultures gave low H_2 uptake rates. High hydrogenase activity in liquid cultures was obtained by incubating suspensions of cells in Mg-phosphate buffer under a gas phase composed of 86.7% N_2, 8.3% H_2, 4.2% CO_2, and 0.8% O_2. Under these conditions the kinetics of hydrogenase induction were easily monitored (Fig. 1). Hydrogenase activity was first detected approximately 3 h after addition of H_2 to the suspension, and activity continued to increase at a rapid rate until 21 h (data points beyond 15 h are not shown). This method for derepressing hydrogenase did not result in a detectable level of nitrogenase activity. Consequently, the expression of hydrogenase activity was dependent upon the addition of exogenous H_2 during the derepression period (Table 1). Cultures in-

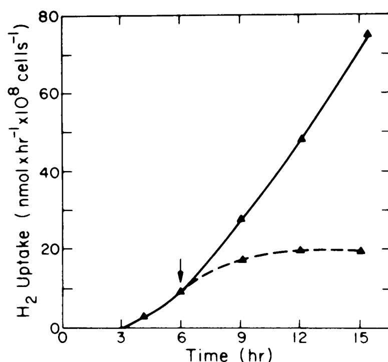

FIG. 1. *Effect of removal of H_2 on hydrogenase induction. Cultures were grown and then derepressed for 6 h (arrow) in an atmosphere of 86.7% N_2, 8.3% H_2, 4.2% CO_2, and 0.8% O_2 as described in the text. Each point in the figure represents an independent H_2 uptake assay of 6 ml of cells from one bottle. At 6 h each bottle was flushed with N_2 (arrow). Gases were then injected into the bottles to obtain an atmosphere composed of 86.7% N_2, 8.3% H_2, 4.2% CO_2, and 0.8% O_2, and the time courses of H_2 uptake was monitored* (▲——▲). *After flushing with N_2 at 6 h another series of cultures received gases to obtain an atmosphere composed of 86.7% N_2, 8.3% Ar, 4.2% CO_2, and 0.8% O_2, and H_2 uptake rates were monitored* (▲- - - -▲).

TABLE 1. *Effect of H_2, CO_2, rifampin, and chloramphenicol on hydrogenase induction*

Conditions of derepression	Hydrogenase activity (nmol of $H_2 \times h^{-1} \times 10^8$ cells^{-1})
Complete, H_2, CO_2, low O_2[a]	64 ± 5
H_2 omitted	0
CO_2 omitted	30 ± 3
Complete plus rifampin	0
Complete plus chloramphenicol	0

[a] Cells were cultured, collected, and derepressed for 15 h in the presence of a gas phase composed of (complete condition) 86.7% N_2, 8.3% H_2, 4.2% CO_2, and 0.8% O_2 as described in the text. Rates of H_2 uptake were determined as described in the text. Results are mean ± standard error of the mean for three independent cultures. The minimum detectable quantity of H_2 oxidized was estimated as 0.5 nmol $\times h^{-1} \times 10^8$ cells^{-1} and values below this are reported as zero. Both rifampin and chloramphenicol were added at a concentration of 25 μg/ml. When H_2 or CO_2 was omitted from the gas phase sufficient Ar was added to obtain 1 atmosphere.

cubated under conditions comparable with those mentioned above, with the exception that Ar replaced H_2 in the atmosphere, showed no hydrogenase activity. In addition to the requirements for H_2 and a low O_2 partial pressure during derepression, the addition of CO_2 at the same time that H_2 was added to initiate derepression resulted in a stimulation of hydrogenase activity (Table 1). In all experiments therefore, 4.2% CO_2 was added to the gas phase above cultures for the entire induction period.

Previously we observed that it was necessary to preincubate non-N_2-fixing cultures of *R. japonicum* with H_2 to demonstrate H_2 uptake activity (12). To investigate the apparent induction of the hydrogenase system by H_2, the effect of the addition and removal of H_2 on expression of hydrogenase activity was examined (Fig. 1). When H_2 was removed after 6 h of induction, further increase in hydrogenase activity ceased within 3 h, indicating an H_2 requirement for hydrogenase induction. The addition of rifampin or chloramphenicol to cells subjected to derepressing conditions resulted in no detectable hydrogenase activity (Table 1). The incubation of previously induced cultures with rifampin or chloramphenicol, however, caused no decrease in hydrogenase activity. From these results we conclude that H_2 does not regulate hydrogenase by activation of a preformed component of the hydrogenase system but is required for hydrogenase synthesis.

Repression by organic compounds. It is well known that organic compounds repress hydrogenase in the H_2-oxidizing bacteria (17). The greatest hydrogenase activities were observed in cultures of *R. japonicum* grown on a medium with a low concentration of carbon substrates (12). Therefore several carbon sources were tested for their ability to repress hydrogenase, and all carbon compounds tested, with the exception of sorbitol, completely repressed hydrogenase formation (Table 2). No growth of *R. japonicum* was observed, however, on a medium identical to that described (2), with the exception that sorbitol was substituted for mannitol. Repression by glutamate was probably associated with its utilization as a source of carbon rather than N, since neither KNO_3 or NH_4Cl repressed hydrogenase formation (Table 2).

The possibility must be considered that carbon sources directly affected hydrogenase by competing for electron carriers. To examine this possibility the organic compounds of Table 2 were added to cells that were actively oxidizing H_2. Experiments of this type conducted with the amperometric device showed no direct effect of the components listed in Table 2 on H_2 oxidation. Therefore, the carbon sources used do not appear to directly or rapidly inhibit hydrogenase activity. The addition of some of the organic compounds to H_2-utilizing cultures caused an immediate increase in O_2 uptake rates (determined amperometrically). These organic compounds therefore were utilized by the cells. The addition of 15 mM succinate, for example, resulted in an immediate twofold increase in O_2 uptake rate, yet no change in the H_2 uptake rate was detected.

The repressing effect by carbon substrates was further investigated in experiments in which carbon compounds were added 6 h after the beginning of hydrogenase induction. For the induction experiments, cells were cultured in a medium containing arabinose and gluconate and then were removed and suspended in Mg-phosphate buffer, and H_2 was added to initiate induction. In tests of the effect of adding arabinose after 6 h of derepressing conditions, hydrogenase induction continued for about 3 h before ceasing (Fig. 2). An effect similar to that obtained from the addition of arabinose was observed when sodium gluconate was added at 6 h. Both carbon sub-

TABLE 2. *Effect of carbon and nitrogen sources on H_2-mediated derepression of hydrogenase in R. japonicum*[a]

Addition	Hydrogenase activity (nmol of H_2 × h^{-1} × 10^8 cells^{-1})
None	55
Sodium gluconate	0
L-Arabinose	0
Sodium citrate	0
Sodium glutamate	0
Sodium succinate	0
Glycerol	0
Sucrose	0
Sorbitol	51
Potassium nitrate (10 mM)	60
Ammonium chloride (10 mM)	58

[a] Portions (5 ml) of cells in Mg-phosphate buffer (approximately 4 × 10^8 cells per ml) were added to 30-ml serum vials. The carbon source at pH 7.0 in the Mg-phosphate buffer was added to the vial at a final concentration of 15 mM. After 30 min of incubation with the carbon source, the vials were stoppered and flushed thoroughly with N_2, and gases were injected to obtain an atmosphere of 84% N_2–10% H_2–5% CO_2–1% O_2. After 15 h, the vials were flushed with N_2 and assayed amperometrically for H_2 uptake activity as described in the text. The minimum detectable rate of H_2 uptake was judged to be 0.5 nmol × h^{-1} × 10^8 cells^{-1}. Cells were grown on agar medium as described previously (12) and washed off the medium in Mg-phosphate buffer as described in the text.

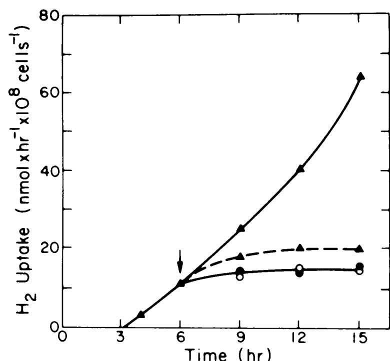

FIG. 2. *Effect of addition of arabinose, oxygen, and rifampin on hydrogenase induction. Cultures were grown and derepressed as described in the text. Each point in the figure represents an independent H_2 uptake assay of 6 ml of cells from one bottle. After 6 h the repressor was added (arrow), and amperometric H_2 uptake assays were conducted at 9, 12, and 15 h. Symbols ▲——▲, No addition at 6 h; ▲- - - -▲, addition of 15 mM arabinose in Mg-phosphate buffer at 6 h; ●——●, O_2 partial pressure increased to 20% at 6 h; ○——○, addition of rifampin (25 μg/ml) at 6 h. The repression effect from addition of sodium gluconate (15 mM) was similar to that obtained from adding arabinose. Results reported are typical of those obtained in three additional experiments.*

strates repressed induction, but the repression failed to occur immediately after their addition.

Repression by O_2. Since greater H_2 uptake rates were observed when *R. japonicum* was grown under decreased (1%) O_2 partial pressures (12), O_2 was tested as a possible repressor of hydrogenase synthesis. When the O_2 partial pressure in the atmosphere was increased to 20% in bottles containing cells previously induced for 6 h, hydrogenase induction ceased abruptly (Fig. 2). The addition of either rifampin or chloramphenicol to suspensions after the induction was initiated resulted in inhibition of enzyme formation similar to that observed by O_2 repression (Fig. 2). Control experiments ruled out the possibility that the addition of O_2 to H_2-utilizing suspensions inactivated the hydrogenase system.

Effect of CO_2. When the optimum conditions for induction of hydrogenase were established, a twofold stimulation of hydrogenase activity was obtained when 4.2% CO_2 was added to the gas phase above cultures (Table 1). It is possible that added CO_2 was needed for maximum expression of hydrogenase activity in the CO_2-deficient culture. To test for a possible direct effect of CO_2 on H_2 uptake rates, cells were induced for hydrogenase activity in the absence of added CO_2, then monitored for rates of H_2 uptake in the presence and absence of added CO_2. The addition of CO_2 to suspensions of cells from these cultures during a 30-min assay in the amperometric device had no effect on hydrogenase activity. Therefore, there seems to be no direct effect of CO_2 on the rate of H_2 uptake.

To study the effect of CO_2 on hydrogenase formation, CO_2 was removed from cultures which previously had been induced in the presence of H_2 and CO_2. Hydrogenase activity was then monitored periodically after CO_2 removal. Removal of CO_2 from the cultures resulted in a decrease in the rate of hydrogenase formation when activities were based upon either cell number (Fig. 3) or protein content. Added CO_2 apparently stimulated hydrogenase formation. To test the hypothesis that CO_2 exerted its effect at the level of enzyme synthesis, CO_2 and chloramphenicol together were added to induced cultures at 6 h, and then hydrogenase activity was measured at 15 h. Hydrogenase activity after 15 h of induction was essentially the same as that measured at 6 h (data not shown). Since a CO_2 requirement for growth of several *Rhizobium* species has been demonstrated (10), the stimulatory effect of CO_2 could be due to some unidentified positive physiological effect on cellular

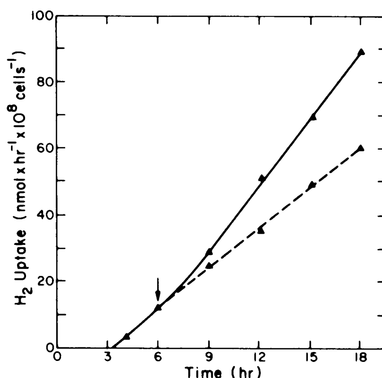

FIG. 3. *Effect of CO_2 removal on hydrogenase induction. Cultures were grown and derepressed as described in the text. Each point in the figure represents an independent H_2 uptake assay of 6 ml of cells from one bottle. After 6 h of derepressing conditions each bottle was flushed with N_2 (arrow). Gases were injected to make an atmosphere composed of 86.7% N_2, 8.3% H_2, 4.2% CO_2, and 0.8% O_2, and H_2 uptake rates were monitored (▲——▲). After flushing with N_2 at 6 h another series of cultures received 5 ml of 5% NaOH (added to a test tube [10 by 100 mm] inside the bottle and containing a filter paper wick). The gas phase in these cultures was the same as listed above except that Ar replaced CO_2. H_2 uptake rates in these cultures were then monitored (▲- - - -▲). Results presented are typical of those obtained in three independent experiments.*

metabolic processes rather than to a specific effect on hydrogenase formation. Oxgen uptake rates were compared, therefore, in cultures derepressed in the presence and in the absence of CO_2. Cultures derepressed in the presence of added CO_2 exhibited no greater rates of O_2 uptake than those derepressed without added CO_2.

DISCUSSION

The results clearly demonstrate that H_2 is required for the synthesis of some essential components of the H_2 uptake system in *R. japonicum.* The removal of H_2 or the addition of H_2 plus rifampin or chloramphenicol to hydrogenase-depressed cultures terminated further increases in hydrogenase activity. Whether H_2 per se or some metabolite resulting from H_2 oxidation (such as ATP) is needed for synthesis of the H_2 uptake sustem has not been established. Oxidizable substrates such as succinate repressed hydrogenase synthesis and, therefore, their capacity to provide energy for support of hydrogenase synthesis could not be determined. When *R. japonicum* was grown under conditions where nitrogenase was not detected, a preincubation period in the presence of H_2 was required to obtain hydrogenase activity (12). Since an active nitrogenase system in cells produces H_2 as a by-product, the induction of the H_2 uptake system in *Rhizobium* may be indirectly regulated by nitrogenase activity.

The repression effect on hydrogenase in *R. japonicum* by organic compounds is similar to that observed for hydrogenase repression in autotrophic H_2-oxidizing bacteria (1, 17). All usable carbon sources tested repressed hydrogenase formation. The organic compounds did not appear to directly inhibit hydrogenase activity, but inhibited synthesis of hydrogenase. The synthesis of hydrogenase in *R. japonicum* as well as in autotrophic H_2-oxidizing bacteria (17) could be controlled by factors related to the utilization of organic compounds, such as the cellular pool size of ATP or NADH. Since sucrose and organic acids repressed hydrogenase formation in free-living cultures, it is of interest to note that these organic compounds are major photosynthetic products translocated from the nodules of soybeans (3).

Oxygen strongly repressed hydrogenase synthesis in *R. japonicum* but did not inhibit activity of the enzyme in whole cells. It was consistently observed that addition of 20% O_2 caused a more rapid rate of repression than addition of arabinose. It appears, therefore, that the mechanism of repression by carbon substrates is different from that by O_2. It is not known whether O_2 per se or a secondary factor resulting from O_2 exposure is the actural repression effector. The rapid repressive effects of O_2, and inhibition of synthesis by rifampin and chloramphenicol are all similiar, indicating that new proteins produced in response to O_2 are not needed for hydrogenase repression. The repression characteristics by O_2 on hydrogenase in *R. japonicum* are analogous to those by O_2 on nitrogenase synthesis in *Klebsiella pneumoniae* (15). In contrast, the repression by O_2 on hydrogenase induction in the hydrogen bacterium *Aquaspirillum autotrophicum* is slow; hydrogenase activity continued to increase after O_2 addition although at a diminished rate (1).

Carbon dixoide is somehow involved in the stimulation of synthesis of the H_2 uptake system in *R. japonicum.* Stimulation of H_2 uptake by added CO_2 has been observed in some autotrophic H_2-oxidizing bacteria (4, 16, 17) and in a *Rhizobium trifolii* mutant (11). The CO_2 enhancement (known as Bartha effect) on hydrogenase in the autotrophic H_2-oxidizing bacteria presumably has been attributed to a direct enhancement of hydrogenase activity (16, 17). In free-living *R. japonicum* however, the addition of CO_2 does not directly stimulate H_2 uptake and does not activate preformed hydrogenase. The stimulation of hydrogenase formation by CO_2 and the observation that increased CO_2 fixation occurred when free-living *R. japonicum* was induced for hydrogenase (H. J. Evans et al., Proceedings of a Workshop Conference, Gottingen, W. Germany, in press) indicates that some interrelationship exists between hydrogenase synthesis and CO_2 fixation in *R. japonicum.*

The regulation of hydrogenase formation in free-living *R. japonicum* includes induction by H_2, repression by organic compounds and O_2, and stimulation of induction by added CO_2. The regulation of hydrogenase synthesis, therefore, is complex and may prove to be important in the control of activity within the bacteroids where hydrogenase may play an important physiological role in nodule metabolism. Now that mutant strains of *R. japonicum* have been obtained that lack ability to utilize H_2 [R. J. Maier, J. R. Postgate, and H. J. Evans, Nature (London), in press; R. J. Maier, Proceedings of the Steenbock-Kettering International Symposium on N_2 Fixation, Abstr. no. C75 1978] their characterization should aid in our understanding of the biochemical factors involved in the H_2 uptake system and perhaps hydrogenase regulation.

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LITERATURE CITED

1. **Aragno, M., and H. G. Schlegel.** 1978. Physiological characterization of the hydrogen bacterium *Aquaspirillum autotrophicum*. Arch. Microbiol. **116**:221-229.
2. **Bergersen, F. J.** 1961. The growth of *Rhizobium* in synthetic media. Aust. J. Biol. Sci. **14**:349-360.
3. **Bergersen, F. J.** 1971. Biochemistry of symbiotic nitrogen fixation in legumes, p. 121-140. *In* L. Machlis, W. Briggs, and R. Park (ed.), Annual review of plant physiology. Annual Review Inc., Palo Alto, Calif.
4. **Bongers, L.** 1970. Energy generation and utilization in hydrogen bacteria. J. Bacteriol. **104**:145-151.
5. **Carter, K. R., N. T. Jennings, F. J. Hanus, and H. J. Evans.** 1978. Hydrogen evolution and uptake by nodules of soybeans inoculated with different strains of *Rhizobium japonicum*. Can. J. Microbiol. **24:307-311.**
6. **Dixon, R. O. D.** 1972. Hydrogenase in legume root nodule bacteroids: occurrence and properties. Arch. Mikrobiol. **85**:193-201.
7. **Emerich, D. W., T. Ruiz-Argüeso, T.-M. Ching, and H. J. Evans.** 1979. Hydrogen-dependent nitrogenase activity and ATP formation in *Rhizobium japonicum* bacteroids. J. Bacteriol. **137**:153-160.
8. **Evans, H. J., T. Ruiz-Argüeso, N. T. Jennings, and F. J. Hanus.** 1977. Energy coupling efficiency of symbiotic nitrogen fixation, p. 333-354. *In* A. Hollaender (ed.), Genetic engineering for nitrogen fixation. Plenum Publishing Corp., New York.
9. **Kuykendall, L. D., and G. H. Elkan.** 1976. *Rhizobium japonicum* derivatives differing in nitrogen-fixing efficiency and carbohydrate utilization. Appl. Environ. Microbiol. **32**:511-519.
10. **Lowe, R. H., and H. J. Evans.** 1962. Carbon dioxide requirement for growth of legume nodule bacteria. Soil Sci. **94**:351-356.
11. **McCrae, R. E., F. J. Hanus, and H. J. Evans.** 1978. Properties of the hydrogenase system in *Rhizobium japonicum* bacteroids. Biochem. Biophys. Res. Comm. **80**:384-390.
12. **Maier, R. J., N. E. R. Campbell, F. J. Hanus, F. B. Simpson, S. A. Russell, and H. J. Evans.** 1978. Expression of hydrogenase in free-living *Rhizobium japonicum*. Proc. Natl. Acad. Sci. U.S.A. **75**:3258-3262.
13. **O'Gara, F., and K. T. Shanmugam.** 1978. Mutant strains of clover rhizobium (*Rhizobium trifolii*) that form nodules on soybeans (*Glycine max*). Proc. Natl. Acad. Sci. U.S.A. **75**:2343-2347.
14. **Rivera-Ortiz, J. M., and R. H. Burris.** 1975. Interactions among substrates and inhibitors of nitrogenase. J. Bacteriol. **123**:537-545.
15. **St. John, R. T., V. K. Shah, and W. J. Brill.** 1974. Regulation of nitrogenase synthesis by oxygen in *Klebsiella pneumoniae*. J. Bacteriol. **119**:266-269.
16. **Schink, B., and H. G. Schlegel.** 1978. Mutants of *Alcaligenes eutrophus* defective in autotrophic metabolism. Arch. Microbiol. **117**:123-129.
17. **Schlegel, H. G., and U. Eberhardt.** 1972. Regulatory phenomena in the metabolism of knallgas bacteria, p. 205-242. *In* A. H. Rose and D. W. Tempest (ed.), Advances in microbial physiology, vol. 7. Academic Press Inc., New York.
18. **Schubert, K. R., and H. J. Evans.** 1976. Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc. Natl. Acad. Sci. U.S.A. **73**:1207-1211.
19. **Schubert. K. R., N. T. Jennings, and H. J. Evans.** 1978. Hydrogen reactions of nodulated leguminous plants. II. Effects on dry matter accumulation and nitrogen fixation. Plant Physiol. **61**:398-401.
20. **Wang, R., F. P. Healy, and J. Meyers.** 1971. Amperometric measurement of hydrogen evolution in *Chlamydomonas*. Plant Physiol. **48**:108-110.