Uptake Hydrogenase Activity in Denitrifying *Azospirillum brasilense* Grown Anaerobically with Nitrous Oxide or Nitrate

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Azospirillum brasilense Sp7 was grown anaerobically with N_2O as the terminal electron acceptor and NH_4Cl as the nitrogen source. Hydrogen uptake activity (O_2-dependent H^3H oxidation) was expressed in the presence and absence of 5% H_2; it reached its maximum in late logarithmic phase as the malate became limiting. This activity was very stable in stationary phase, even in the absence of exogenous H_2, compared with microaerobically grown cultures; this supports the hypothesis that the exclusion of O_2 is critical for maintaining the integrity of the H_2 uptake system in this organism. Oxygen, as well as methylene blue and N_2O, supported H_2 uptake, indicating the presence of electron transport components leading to O_2 in anaerobically grown *A. brasilense*. Nitrite (0.5 mM) inhibited H_2 uptake. In cultures grown with NO_3^- as the terminal electron acceptor and NH_4Cl as the nitrogen source, in the presence and absence of exogenous H_2, only low H_2 uptake activity was observed. Methylene blue, O_2, N_2O, NO_3^-, and NO_2^- were all capable of acting as the electron acceptor for H_2 oxidation. Nitrite (0.5 mM) did not inhibit H_2 uptake in NO_3^--grown cells, as it did in N_2O-grown cells. *A. brasilense* appears to be one of the few organisms capable of expressing the H_2 uptake system under denitrifying conditions in the absence of molecular H_2.

Azospirillum brasilense, formerly *Spirillum lipoferum* (23), is a widespread soil organism in tropical regions (8) and may be the major bacterium responsible for nitrogenase activity in several tropical grass associations (7). It possesses a respiratory type of metabolism and participates in several major nitrogen transformations. *A. brasilense* fixes N_2 under microaerobic conditions (6, 15, 19), denitrifies under anaerobic conditions (9, 15, 16), and assimilates NH_4^+, NO_3^- (6), or NO_2^- (16).

Nelson and Knowles (15) established anaerobic, denitrifying steady-state continuous cultures with NO_3^- as the electron acceptor and sole nitrogen source for growth. Bothe et al. (1) observed growth in NO_3^--containing batch cultures but were unable to observe substantial growth with NO_2^-. Recently, R. Lalande and R. Knowles (Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, N66, p. 189) demonstrated that *A. brasilense* could grow anaerobically with NO_3^-, NO_2^-, or N_2O as the terminal electron acceptor and NH_4Cl as the nitrogen source. In NO_3^--grown cultures, NO_2^- and N_2O were sequentially produced and reduced. The presence of NO_3^- (0.18 mM) in the medium was necessary for subsequent growth on NO_2^- and shortened the lag phase in N_2O-grown cultures by several hours.

A. brasilense has an uptake hydrogenase with more than sufficient activity to recycle nitrogenase-produced H_2 (5) and is capable of H_2-dependent C_2H_2 reduction (20). The H_2-dependent respiration is very O_2-sensitive (20), but the O_2 optimum for the H_2 uptake system is significantly higher than that for nitrogenase activity (23a), suggesting that H_2 uptake may have a limited ability to aid in the protection of nitrogenase against inactivation by O_2. In *A. brasilense* hydrogenase derepression requires microaerobic conditions, is independent of nitrogenase derepression since it is expressed in NH_4Cl-grown cultures, does not require exogenous H_2, and may be enhanced by electron donor limitation (23a).

Since organisms such as *Paracoccus denitrificans*, *Rhizobium japonicum*, and *Alcaligenes eutrophus* possess hydrogenase activity under denitrifying conditions (11, 13, 21) and because *A. brasilense* shows both hydrogenase activity and the ability to denitrify, it was our objective to determine whether hydrogenase was expressed under denitrifying conditions in *A. brasilense*. We report the presence of high levels of H_2 uptake in N_2O-grown cultures but only low levels in NO_3^--grown cultures.

MATERIALS AND METHODS

Bacterial strain, growth medium, and culture conditions. *A. brasilense* Sp7 (ATCC 29145) was grown in the defined medium previously described (15) and modified (23a). The medium was supplemented with 7.2 mg liter^{-1} of KNO_3 (71 μM) and 50 mg liter^{-1} of KNO_3 (0.5 mM) for N_2O-grown and NO_3^--grown cultures, respectively. Stock cultures and culture conditions were as previously described (23a), except that the batch cultures were sparged at 240 ml min^{-1}, and the gas mixtures were 10% N_2O in N_2 (Liquid Carbonic Canada, St. Laurent, Quebec) with or without 5% H_2 and N_2 with or without H_2 for N_2O-grown and NO_3^--grown cultures, respectively.

Uptake hydrogenase assay. Oxygen-dependent H_2 uptake was measured by the H^3H oxidation method, as previously described (23a), in 50-ml Erlenmeyer flasks with 5-ml culture samples. Unless otherwise indicated, 1% O_2, 2.6% H_2, and 0.4% H^3H were added to start the 15-min assays. One percent O_2 (11.8 μM) was chosen for the standard assay because it is near the optimum for H_2 uptake in most phases of growth (23a). This was verified for N_2O-grown and NO_3^--grown cultures. When alternative electron acceptors were used, methylene blue, KNO_3, and KNO_2 solutions were kept in serum-stoppered containers and made anaerobic by evacuating and refilling them with N_2 to 1 atm (101.3 kPa) three times. Aliquots were transferred to assay flasks by syringe. The H^3H (specific activity, 17.5 μCi ml^{-1}) was obtained and stored in a lecture bottle (Matheson Canada,

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Whitby, Ontario). Air-incubated cells were used as blanks, and these values were subtracted from those obtained from other treatments. The small amount of H^3H uptake by cells incubated under air cannot be lowered by first autoclaving the cells, and the inhibition due to the O_2 present in air is irreversible (23a). Since there is no significant difference between the rates of H^3H and H_2 uptake by A. brasilense when using O_2 (23a) or methylene blue (unpublished data) as the electron acceptor, H^3H uptake appears to be a valid measure of H_2 oxidation in this organism.

Oxidized methylene blue is assumed to accept electrons directly from hydrogenase and has been used to measure hydrogenase activity specifically (20), whereas O_2-dependent H_2 uptake measures the activity of the H_2 oxidation system as a whole. Results to be presented, and unpublished data, show that there is usually no more than a twofold difference between the activities with 5 mM methylene blue and those obtained with 1% O_2. Since methylene blue-dependent H_2 uptake has an optimum for methylene blue (5 mM) and higher concentrations inhibit the activity (data not shown), this method shows some of the same problems as are found with the use of O_2. Therefore, for A. brasilense we have used O_2-dependent H_2 uptake as a means of estimating hydrogenase activity, since at an optimal concentration of O_2 it closely approximates the results obtained with methylene blue.

Analyses. Oxygen, N_2O, and H_2 were measured by gas chromatography as previously described (2, 4, 23a). Culture samples were taken at specified times for absorbance readings at 430 nm with a Bausch & Lomb Spectronic 20 and then frozen. The samples were thawed and used for Lowry protein estimation and the enzymatic determination of L-malic acid as described previously (23a). Cells were removed by membrane filtration (0.22-μm pore size), and NO_3^- and NO_2^- levels were determined by the Griess-Ilosvay method (by using sulfanilamide reagent with and without hydrazine-copper reduction) with an automated analysis system (ChemLab Instruments, Hornchurch, Essex, England). Spot tests for NO_3^- and NO_2^- (by using drops of sulfanilamide reagent with and without zinc reduction) were carried out on 0.5-ml culture samples. Ostwald coefficients (26) were used to calculate the concentrations of gases in solution. All data are expressed as the means of duplicate samples except for H^3H uptake assays, which are the means of triplicate flasks.

RESULTS

Uptake hydrogenase in N_2O-grown cultures. A. brasilense was grown anaerobically with N_2O (10% vol/vol in the gas phase, 2 mM in solution) as the terminal electron acceptor and NH_4Cl as the nitrogen source (Fig. 1). A small amount of NO_3^-, present initially to shorten the lag phase, was reduced to NO_2^-, and this in turn was completely used within 6 h. After this initial period, N_2O alone supported the terminal oxidant needs of the cultures with a generation time of 3 h. This compared favorably with generation times observed in cultures grown microaerobically on NH_4Cl (3 to 3.5 h) and under N-free conditions (5.5 h) (23a) and aerobically on NH_4Cl (1 to 2 h) (18). Malate, the carbon and energy source, was the growth-limiting component since growth, measured by absorbance (data not shown) and protein content, ended when the malate was fully depleted. We observed high levels of H_2 uptake, reaching a maximum in late log phase, in the presence and absence of exogenous H_2. Hydrogen uptake activity in the $+H_2$ culture reached a higher maximum and was more stable in stationary phase than in the $-H_2$ culture.
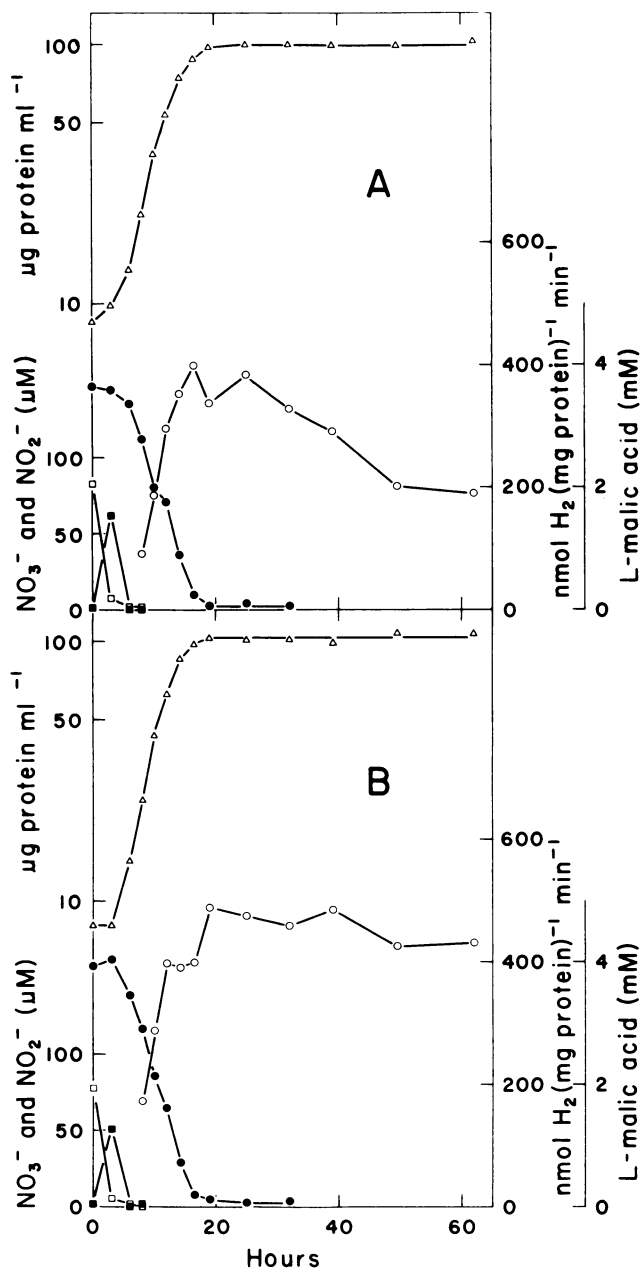

FIG. 1. Hydrogen uptake activity in N_2O-grown cultures in the absence (A) and presence (B) of 5% H_2. Symbols: ○, O_2-dependent H_2 uptake; △, protein; ●, L-malic acid; □, NO_3^-; ■, NO_2^-.

Although the final protein content of the two cultures was nearly the same, the maximum absorbance (not shown) of the $+H_2$ culture was higher and more stable in stationary phase than that of the $-H_2$ culture (0.66 versus 0.58, dropping to 0.53). This absorbance difference may reflect some changes in cell content, size, and morphology due to the presence of H_2, and the drop in absorbance may be due to cell lysis. The initial and final pH values, about 6.7 and 7.1, respectively, were similar in both cultures.

With N_2O-grown cells, methylene blue, O_2, and N_2O could each serve as the electron acceptor for H_2 uptake, whereas NO_2^- inhibited H_2 uptake (Table 1).

Uptake hydrogenase in NO_3^--grown cultures. *A. brasilense* was grown anaerobically with NO_3^- (0.5 mM initially) as the terminal electron acceptor and NH_4Cl as the nitrogen source (Fig. 2). The NO_3^- was depleted, and NO_2^-, the reduction product, reached its maximum by 9 h. The NO_2^- maximum corresponds to a shoulder or plateau in the absorbance (not shown), protein, and malate curves. This phenomenon has been observed repeatedly in other NO_3^--grown cultures (Lalande and Knowles, unpublished data). The duration of the shoulder or plateau was dependent upon the initial concentration of NO_3^-; the more NO_3^- added, the more NO_2^- produced and the longer the interruption of growth. By 11 h (Fig. 2), NO_2^- reduction had begun, and the cells grew with NO_2^- as the terminal electron acceptor. Although not shown here, in similar cultures, concomitant with the reduction of NO_2^- was the release of N_2O, which was detected in the effluent gas from the cultures. By 14 h NO_2^- had disappeared and growth had stopped. At 16.5 h, and thereafter whenever NO_2^- could not be detected in the cultures by spot test, spikes of NO_3^- (yielding a final concentration of 0.4 to 0.5 mM) were added to the cultures to allow growth to continue.

We detected only low levels of H_2 uptake in NO_3^--grown cultures, in the presence and absence of exogenous H_2 (Fig. 2). These levels were roughly 50 to 100 times lower than those in N_2O-grown cultures. What little activity there was appeared to reach a maximum as the malate disappeared and was more stable and roughly two times higher in the $+H_2$ culture than in the $-H_2$ culture.

After 52 h there was no malate remaining in the cultures. A further slow reduction of NO_3^- took place, but only in the $+H_2$ culture (Fig. 2). Protein and absorbance values for NO_3^--grown cultures in the presence and absence of exogenous H_2 were nearly the same; maximum absorbance values (not shown) were 0.423 and 0.440, respectively. The initial and final pH values, about 6.7 and 7.4, respectively, were similar in both cultures.

For NO_3^--grown cells, methylene blue, O_2, N_2O, NO_3^-, and NO_2^- were all capable of acting as the electron acceptor for H_2 uptake (Table 2). Hydrogen uptake was not inhibited by 0.5 mM NO_2^-, in contrast to N_2O-grown cells.

DISCUSSION

A. brasilense appears to be one of the few organisms capable of synthesizing hydrogenase under denitrifying con-
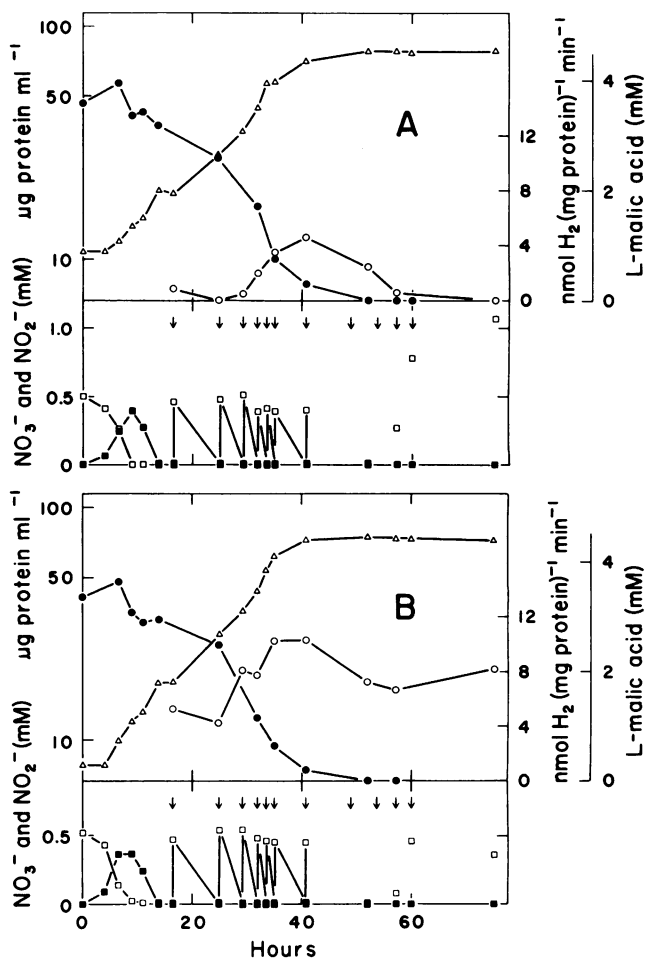

FIG. 2. Hydrogen uptake activity in NO_3^--grown cultures in the absence (A) and presence (B) of 5% H_2. Arrows indicate the times at which NO_3^- spikes were added. Samples for NO_3^- and NO_2^- analysis were taken both before and after the NO_3^- spike up to and including 41 h; but after 41 h the samples were taken only before the spike, at the same time as cells were removed for H_2 uptake assays. Symbols as in the legend to Fig. 1.

TABLE 1. Uptake hydrogenase activity of N_2O-grown *A. brasilense* with different electron acceptors[a]

Electron acceptor (concn)	H_2 uptake[b]	Relative activity (%)
Methylene blue (5.0 mM)	463.5 ± 24.2	100.0
0.5% O_2 (5.9 μM)	270.5 ± 8.0	58.4
1.0% O_2 (11.8 μM)	424.7 ± 1.0	91.6
N_2O (1.0 mM)	70.2 ± 1.2	15.1
NO_3^- (0.5 mM)	28.8 ± 5.4	6.2
NO_2^- (0.5 mM)	6.7 ± 0.6	1.4
None added	32.0 ± 1.5	6.9

[a] Late-log-phase culture grown in the absence of H_2. Protein content was 71.8 μg of protein ml^{-1}. Before samples were taken, the culture was purged of N_2O by flushing with N_2 for 15 min. Flasks with culture samples were then preincubated for 30 min to allow the cells to use any contaminating N_2O or O_2 (verified by gas chromatography).

[b] Activities expressed as nmol of H_2 (mg of protein)$^{-1}$ min^{-1}. Assays were 24 min long. Results are means of three flasks ± standard error.

ditions in the absence of molecular H_2. Others include *P. denitrificans* (17) and *A. eutrophus* (21). Hydrogenase expression in *P. denitrificans* strains other than 381 appears to be constitutive (17) and in *A. eutrophus* is associated with electron donor limitation (10). *R. japonicum* possesses hydrogenase activity under denitrifying conditions (13) but requires the presence of H_2 for hydrogenase expression (12).

In N_2O-grown cultures the high rates of H_2 uptake observed reached a maximum as the malate (electron donor) became limiting. Hydrogen was not necessary for hydrogenase derepression but increased the maximum activity and stability of the H_2 uptake system. In cultures grown under microaerobic conditions in the absence of H_2 there is a rapid decay of H_2 uptake activity in stationary phase, whereas in the presence of H_2 the H_2 uptake system is much more stable (23a). Hydrogen-dependent respiration is O_2-sensitive (20, 23a), and it seems that in stationary phase, in the absence of an oxidizable substrate, even microaerobic conditions (such as 0.75% O_2) are sufficient to rapidly inactivate the H_2 uptake system (23a). The presence of H_2 in stationary phase

TABLE 2. Uptake hydrogenase activity of NO_3^--grown *A. brasilense* with different electron acceptors[a]

Electron acceptor (concn)	H_2 uptake[b]	Relative activity (%)
Methylene blue (5.0 mM)	17.4 ± 1.7	100.0
0.5% O_2 (5.9 μM)	15.5 ± 2.0	89.1
1.0% O_2 (11.8 μM)	8.6 ± 0.8	49.4
N_2O (1.0 mM)	11.2 ± 3.2	64.4
NO_3^- (0.5 mM)	5.6 ± 1.8	32.2
NO_2^- (0.5 mM)	7.1 ± 2.0	40.8
None added	1.5 ± 1.0	8.6

[a] Stationary-phase culture grown in the presence of 5% H_2. Protein content was 81.6 μg of protein ml^{-1}. Samples were taken when there was no detectable NO_3^- or NO_2^- in the culture, and the flasks were then preincubated for 30 min to remove any contaminating O_2 (verified by gas chromatography).

[b] Activities expressed as nmol of H_2 (mg of protein)$^{-1}$ min^{-1}. Assays were 24 min long. Results are means of three flasks ± standard error.

might allow the H_2 uptake system to protect itself to some extent against O_2-inactivation by H_2-dependent O_2 utilization. Thus, it is not surprising that under anaerobic N_2O-grown conditions, even in the absence of H_2, the H_2 uptake system was relatively stable. In N_2O-grown cultures the increased stability of the H_2 uptake system in the presence of H_2 may be due to the oxidation of H_2 as a source of maintenance energy.

The N_2O-grown cells had higher rates of H_2 uptake when assayed in the presence of O_2 than with N_2O or NO_3^-, and we suggest that electron transport components leading to O_2 are present under anaerobic growth conditions, as in *P. denitrificans* (25).

The higher O_2 optimum for H_2 uptake shown by the late log phase N_2O-grown cells of Table 1 compared with the stationary phase NO_3^--grown cells of Table 2 supports our previous observations with N_2-fixing and NH_4^+-grown *A. brasilense* (23a). The results suggest a limited amount of respiratory protection (with malate) for the H_2 uptake system in late-log-phase culture samples where malate was still present but not for stationary-phase culture samples where malate was no longer present.

Nitrite is toxic to a variety of microorganisms (3, 27), but little is known of the physiological basis for this inhibition. In aerobic organisms it inhibits O_2 uptake, oxidative phosphorylation, and active transport, suggesting that it interferes with electron carriers in the membrane respiratory system (22, 24, 28). Since H_2 oxidation is dependent upon electron transport, it is not surprising that NO_2^- inhibited H_2 uptake in N_2O-grown *A. brasilense*.

From preliminary experiments, usually with 2 mM NO_3^- initially, we had come to expect only low levels of H_2 uptake in NO_3^--grown cultures. However, in these earlier studies, the electron acceptor and not the electron donor was the growth-limiting component. Therefore in the present study we ensured the full depletion of the electron donor (malate) by adding successive NO_3^- spikes. Also, due to the inhibition of H_2 uptake in N_2O-grown cells by NO_2^-, we used a lower concentration of NO_3^- initially (0.5 mM instead of 2 mM) and assayed for H_2 uptake only when spot tests indicated the absence of NO_2^- in the cultures. Despite these modifications, we observed only low H_2 uptake activity in NO_3^--grown cultures. The reasons for this are not clear. Although the initial accumulation of NO_2^- did inhibit growth until NO_2^- reductase was derepressed and reduced the NO_2^- further, the low hydrogenase activity was apparently

not due to inhibition by NO_2^- because NO_2^- supported H_2 oxidation in NO_3^--grown cells. Nitrate-grown cells probably had higher levels of NO_2^- reductase than did N_2O-grown cells because of the constant exposure to NO_2^- in the NO_3^--grown cultures. This might have allowed the NO_3^--grown cells to maintain intracellular NO_2^- concentrations below inhibitory levels.

Nitrate-grown *A. brasilense* used NO_3^-, NO_2^-, N_2O, and O_2 as the terminal electron acceptors for H_2 uptake. This makes it similar to *R. japonicum* grown anaerobically under chemolithotrophic conditions (14). The slow disappearance of NO_3^- and the lack of NO_2^- accumulation in the $+H_2$ culture after the malate was fully depleted were probably due to H_2-dependent NO_3^- and NO_2^- reduction.

The ability of *A. brasilense* to grow using any one of the three major substrates of denitrification and the presence of H_2 uptake activity under these conditions add to the versatility of this organism and help explain its widespread distribution. Further understanding of the relationship between uptake hydrogenase activity and denitrification would be desirable, and we are currently investigating further aspects of the inhibition of H_2 uptake by NO_2^- and other compounds.

LITERATURE CITED

1. **Bothe, H., B. Klein, M. P. Stephan, and J. Döbereiner.** 1981. Transformations of inorganic nitrogen by *Azospirillum* spp. Arch. Microbiol. **130:**96–100.
2. **Brouzes, R., C. I. Mayfield, and R. Knowles.** 1971. Effect of oxygen partial pressure on nitrogen fixation and acetylene reduction in a sandy loam soil amended with glucose, p. 481–494. *In* T. A. Lie and E. G. Mulder (ed.), Biological nitrogen fixation in natural and agricultural habitats. Plant Soil, special volume. Martinus Nijhoff, The Hague, The Netherlands.
3. **Castellani, A. G., and C. F. Niven, Jr.** 1955. Factors affecting the bacteriostatic action of sodium nitrite. Appl. Microbiol. **3:**154–159.
4. **Chan, Y. K., and R. Knowles.** 1979. Measurement of denitrification in two freshwater sediments by an in situ acetylene inhibition method. Appl. Environ. Microbiol. **37:**1067–1072.
5. **Chan, Y. K., L. M. Nelson, and R. Knowles.** 1980. Hydrogen metabolism of *Azospirillum brasilense* in nitrogen-free medium. Can. J. Microbiol. **26:**1126–1131.
6. **Day, J. M., and J. Döbereiner.** 1976. Physiological aspects of N_2-fixation by a *Spirillum* from *Digitaria* roots. Soil Biol. Biochem. **8:**45–50.
7. **Döbereiner, J., and J. M. Day.** 1976. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites, p. 518–538. *In* W. E. Newton and C. J. Nyman (ed.), Proceedings of the 1st International Symposium on Nitrogen Fixation. Washington State University Press, Pullman.
8. **Döbereiner, J., I. E. Marriel, and M. Nery.** 1976. Ecological distribution of *Spirillum lipoferum*. Beijerinck. Can. J. Microbiol. **22:**1464–1473.
9. **Eskew, D. L., D. D. Focht, and I. P. Ting.** 1977. Nitrogen fixation, denitrification, and pleomorphic growth in a highly pigmented *Spirillum lipoferum* Appl. Environ. Microbiol. **34:**582–585.
10. **Friedrich, C. G.** 1982. Derepression of hydrogenase during

limitation of electron donors and derepression of ribulosebis-phosphate carboxylase during carbon limitation of *Alcaligenes eutrophus*. J. Bacteriol. **149**:203–210.

11. **Kluyver, A. J., and W. Verhoeven.** 1954. Studies on true dissimilatory nitrate reduction. IV. On adaptation in *Micrococcus denitrificans*. Antonie van Leeuwenhoek J. Microbiol. Serol. **20**:337–358.

12. **Maier, R. J., F. J. Hanus, and H. J. Evans.** 1979. Regulation of hydrogenase in *Rhizobium japonicum*. J. Bacteriol. **137**:824–829.

13. **Neal, J. L., G. C. Allen, R. D. Morse, and D. D. Wolf.** 1982. Anaerobic nitrate-dependent chemolithotrophic growth by *Rhizobium japonicum*. Can. J. Microbiol. **29**:316–320.

14. **Neal, J. L., G. C. Allen, R. D. Morse, and D. D. Wolf.** 1983. Nitrate, nitrite, nitrous oxide and oxygen-dependent hydrogen uptake by *Rhizobium*. FEMS Microbiol. Lett. **17**:335–338.

15. **Nelson, L. M., and R. Knowles.** 1978. Effect of oxygen and nitrate on nitrogen fixation and denitrification by *Azospirillum brasilense* grown in continous culture. Can. J. Microbiol. **24**:1395–1403.

16. **Neyra, C. A., J. Döbereiner, R. Lalande, and R. Knowles.** 1977. Denitrification by N₂-fixing *Spirillum lipoferum*. Can. J. Microbiol. **23**:300–305.

17. **Nokhal, T.-H., and H. G. Schlegel.** 1980. The regulation of hydrogenase formation as a differentiating character of strains of *Paracoccus denitrificans*. Antonie van Leeuwenhoek J. Microbiol. Serol. **46**:143–155.

18. **Okon, Y., S. L. Albrecht, and R. H. Burris.** 1977. Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl. Environ. Microbiol. **33**:85–88.

19. **Okon, Y., J. P. Houchins, S. L. Albrecht, and R. H. Burris.** 1977. Growth of *Spirillum lipoferum* at constant partial pressures of oxygen, and the properties of its nitrogenase in cell-free ex-tracts. J. Gen. Microbiol. **98**:87–93.

20. **Pedrosa, F. O., M. Stephan, J. Döbereiner, and M. G. Yates.** 1982. Hydrogen-uptake hydrogenase activity in nitrogen-fixing *Azospirillum brasilense*. J. Gen. Microbiol. **128**:161–166.

21. **Pfitzner, J., and H. G. Schlegel.** 1973. Denitrifikation bei *Hydrogenomonas eutropha* Stamm H 16. Arch. Mikrobiol. **90**:199–211.

22. **Rowe, J. J., J. M. Yarbrough, J. B. Rake, and R. G. Eagon.** 1979. Nitrite inhibition of aerobic bacteria. Curr. Microbiol. **2**:51–54.

23. **Tarrand, J. J., N. R. Krieg, and J. Döbereiner.** 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. **24**:967–980.

23a.**Tibelius, K. H., and R. Knowles.** 1983. Effect of hydrogen and oxygen on uptake hydrogenase activity in nitrogen-fixing and ammonium-grown *Azospirillum brasilense*. Can. J. Microbiol. **29**:1119–1125.

24. **Van Verseveld, H. W., E. M. Meijer, and A. H. Stouthamer.** 1977. Energy conservation during nitrate respiration in *Paracoccus denitrificans*. Arch. Microbiol. **112**:17–23.

25. **Vignais, P. M., M.-F. Henry, E. Sim, and D. B. Kell.** 1981. The electron transport system and hydrogenase of *Paracoccus denitrificans*. Curr. Top. Bioenerg. **12**:115–196.

26. **Wilhelm, E., R. Battino, and R. J. Wilcock.** 1977. Low-pressure solubility of gases in liquid water. Chem. Rev. **77**:219–262.

27. **Wodzinski, R. S., D. P. Labeda, and M. Alexander.** 1978. Effects of low concentrations of bisulfite-sulfite and nitrite on microorganisms. Appl. Environ. Microbiol. **35**:718–723.

28. **Yarbrough, J. M., J. B. Rake, and R. G. Eagon.** 1980. Bacterial inhibitory effects of nitrite: inhibition of active transport, but not of group translocation, and of intracellular enzymes. Appl. Environ. Microbiol. **39**:831–834.