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# Aerobic Hydrogen Accumulation by a Nitrogen-Fixing Cyanobacterium, *Anabaena* sp.

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**Hydrogen evolution by a nitrogen-fixing cyanobacterium, *Anabaena* sp. strain N-7363, was tested in order to develop a water biophotolysis system under aerobic conditions. A culture of the strain supplemented with carbon dioxide under an air atmosphere evolved hydrogen and oxygen gas, which reached final concentrations of 9.7 and 69.8%, respectively, after 12 days of incubation. Hydrogen uptake activity was not observed during incubation, and nitrogenase was thought to be the sole enzyme responsible for the hydrogen evolution.**

Nitrogen-fixing cyanobacteria have been studied in many laboratories for use in a system for producing hydrogen from water (2–4, 6–9, 11–18, 20, 21, 23, 25, 26). In considering water biophotolysis by cyanobacteria, one of the problems yet to be solved is the inhibition of hydrogen evolution by nitrogen and oxygen gas. Nitrogen gas is a competitive inhibitor of the hydrogen evolution reaction of nitrogenase, and oxygen gas is also an inactivator of nitrogenase, although aerobic nitrogen-fixers have various protection mechanisms against the inhibitory effect of oxygen gas (5, 10, 22). Therefore, most cyanobacterial hydrogen production systems use argon-based atmospheres.

Moreover, concomitant uptake hydrogenase activity in nitrogen-fixers prevents hydrogen accumulation in closed vessels. Continuous flushing of cyanobacterial cultures with inert gas (15–17, 25) can prevent hydrogen uptake and oxygen inhibition, since the hydrogen and oxygen gases evolved are kept at low concentrations. However, as suggested by Mitsui and co-workers (7, 11), this method is not practical because the hydrogen gas evolved must be separated from a large amount of inert gas. Hydrogen accumulation in a closed system without the use of a noble gas would therefore be advantageous from a practical aspect.

We isolated an aerobic hydrogen-evolver, *Anabaena* sp. strain N-7363 (3), from the culture collection of Nakayama at the University of Yamanashi (19) and demonstrated the prolongation of aerobic and anaerobic hydrogen evolution by this strain in closed vessels in which the gas atmosphere was renewed in a semibatch procedure (2). In this paper, we describe the aerobic evolution and simultaneous accumulation of hydrogen and oxygen gas by *Anabaena* sp. strain N-7363 in a closed vessel supplemented with carbon dioxide without any renewal of the gas atmosphere.

## MATERIALS AND METHODS

*Anabaena* sp. strain N-7363 was cultured in a 300-ml bottle containing 150 ml of modified Allen-Arnon medium free of any combined nitrogen source (3) and $CO_2$-enriched (5%, vol/vol) air at 30°C under continuous illumination of 7 klux (fluorescent lamps) with gentle stirring by a magnetic spin bar. After 5 days, the culture was inoculated into fresh culture medium in a Roux bottle (Fig. 1). The total internal volume of the bottle was 1,550 ml, and the initial working volume was 1,000 ml. The initial gas phase was air enriched with carbon dioxide (5%, vol/vol).

All of the following procedures except step v were repeated every day. (i) The gas samples were withdrawn with a Pressure-Lok syringe (Precision Sampling Corp., Baton Rouge, La.) and applied to a gas chromatograph to deter-

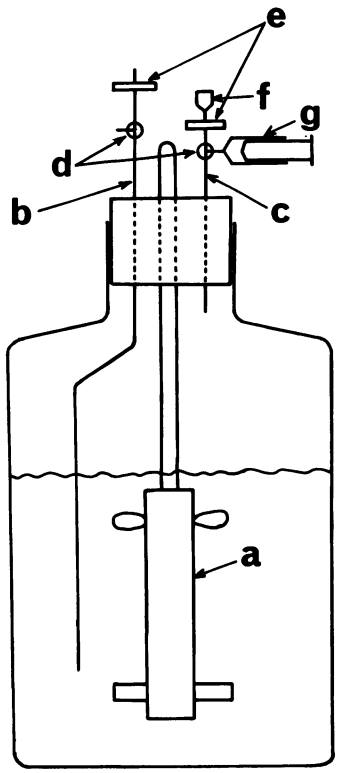


FIG. 1. Incubation vessel. The incubation vessel was a Roux bottle (14 cm wide by 6 cm deep). (a) A Culstar stirring unit (Shibata Glass Co. Ltd., Tokyo, Japan) was adapted and incorporated. (b) Line for withdrawing the culture broth and for $CO_2$ injection (stainless steel needle, 1 mm diameter). (c) Line for gas sampling and measuring internal gas volume (stainless steel needle, 1 mm diameter). (d) Plastic three-way valve (Nipro Co. Ltd., Tokyo, Japan). (e) Membrane filter unit equipped with an FGLP 01300 membrane filter (Millipore Corp., Bedford, Mass.). (f) Gas sampling plug for gas chromatographic determinations. (g) Hypodermic syringe for measuring change of gas volume of the vessel (20 ml in maximal graduation).

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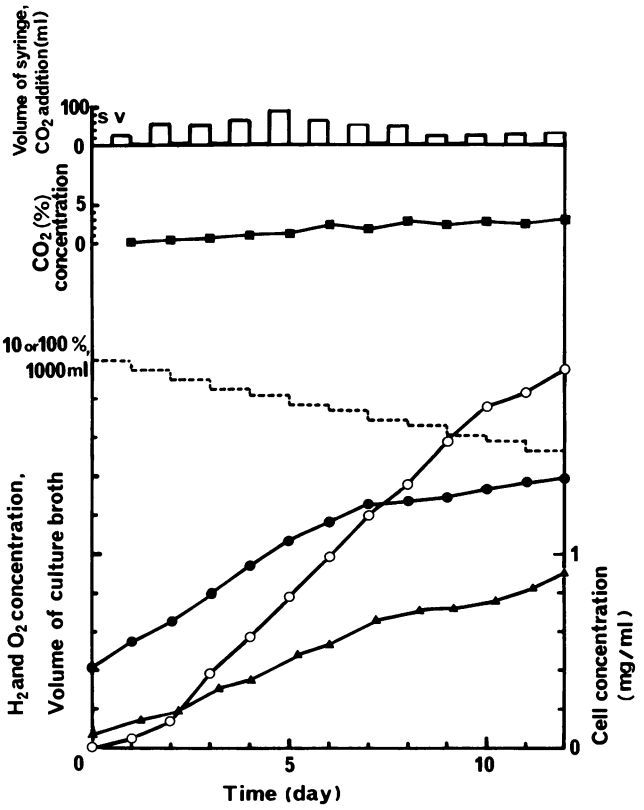


FIG. 2. Time course of hydrogen and oxygen evolution by *Anabaena* sp. strain N-7363. The cell suspension was incubated at 30°C under continuous illumination of 7 klux (fluorescent lamps) in the incubation vessel shown in Fig. 1. The concentrations of $H_2$, $O_2$, and $CO_2$ shown here indicate those determined as described in Materials and Methods. Dashed line indicates the volume of the culture broth. S indicates graduation of the syringe (Fig. 1) measured as described in the text, and V indicates the $CO_2$ volume added as described in the text. The full scale of gas concentration is 10 and 100% for hydrogen and oxygen, respectively. Symbols: ○, hydrogen; ●, oxygen; ■, carbon dioxide; ▲, cyanobacterial cell concentration.

mine the concentrations of hydrogen, oxygen, and carbon dioxide. (ii) After the gas in the vessel was vented into a hypodermic syringe via a three-way valve, the graduation of the syringe was measured at atmospheric pressure and the internal gas in the syringe was discarded. (iii) To restore the concentration of carbon dioxide to 5%, a calculated volume of carbon dioxide was injected and the gas atmosphere was not vented to atmospheric tension until the following day. (iv) A 15-ml amount of the culture was withdrawn to determine the cyanobacterial cell concentration. (v) An additional 10 ml of the culture was withdrawn to assay acetylene-reducing activity and in situ hydrogen uptake in darkness.

The concentration of hydrogen and oxygen gas was determined as reported previously (3). The concentration of carbon dioxide was determined with a Shimadzu GC R1A gas chromatograph (detector; TCD) equipped with an activated charcoal (30/60 mesh) column (3 mm diameter by 2 m). The oven temperature was 140°C and the inlet pressure of the carrier gas (nitrogen) was 1 kg/cm$^2$.

Hydrogen uptake activity in darkness was assayed with a hydrogen-oxygen electrode system (1, 24). Acetylene-reducing activity and cell dry weight were determined as reported previously (1). Hydrogen-evolving activity from reduced methyl viologen was measured with the hydrogen-oxygen electrode. Reduced methyl viologen was prepared as described previously (1) and added at 1 mM (plus 5 mM sodium dithionite) to the cyanobacterial suspensions.

## RESULTS AND DISCUSSION

In the previous paper (reference 2 and Fig. 4 therein), *Anabaena* sp. strain N-7363 was shown to evolve hydrogen gas aerobically when the gas phase was closed by temporal stoppage of aeration. Now we have shown that this strain can accumulate hydrogen and oxygen gas simultaneously even in a completely closed system (Fig. 2).

The conditions for aerobic hydrogen accumulation by intact cells of nitrogen-fixing cyanobacteria in a closed system are as follows: (i) hydrogen evolution is not completely inhibited by nitrogen gas, (ii) the nitrogenase system is highly protected against oxygen gas, and (iii) there is little or no activity of uptake hydrogenase. We have already shown that strain N-7363 can fulfill the first requirement; hydrogen evolution strain by N-7363 was not completely inhibited by nitrogen gas and was possible even in 100% nitrogen gas at a reduced rate (3).

We then examined requirement (ii). Acetylene-reducing activity was measured at various oxygen partial pressures by withdrawing culture samples at various times during hydrogen evolution (Fig. 3). The results indicated that the strain required some oxygen gas for maximal acetylene reduction in every case and that the nitrogenase system of N-7363 was highly resistant against oxygen gas. Although further studies are needed to elucidate this high oxygen resistance, the data in Fig. 3 suggest that respiration may play a dominant role in the protection and reaction of nitrogenase in this strain.

Strain N-7363 may also fulfill requirement (iii) (Table 1). We tried to assay the hydrogen uptake activity of the culture

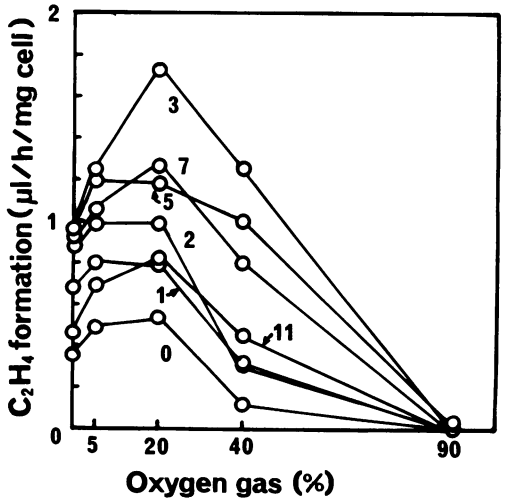


FIG. 3. Acetylene-reducing activity of the samples withdrawn during $H_2$ evolution under various oxygen partial pressures. Each vessel (Erlenmeyer flask), stoppered with a rubber bung, contained 1 ml of culture broth and 6 to 7 ml of gas. The concentration of acetylene gas was 10%. Numbers indicate the day during hydrogen evolution when the sample was withdrawn.

TABLE 1. Hydrogen evolution in situ by *Anabaena* sp. strain N-7363 in darkness[a]

| Sampling time (day) | Concn[b] (%) | | $H_2$ evolution (μl of $H_2$/h per mg of cells [dry wt]) |
|---|---|---|---|
| | $H_2$ | $O_2$ | |
| 1 | 0.40 | 33.2 | 0.371 |
| 2 | 1.02 | 37.7 | 0.765 |
| 3 | 2.18 | 49.5 | 0.581 |
| 5 | 4.18 | 55.0 | 0.533 |
| 7 | 6.92 | 66.6 | 0.539 |
| 11 | 9.70 | 70.4 | 0.216 |

[a] Culture broth which was evolving hydrogen and oxygen as shown in Fig. 2 was withdrawn at the times indicated with a hypodermic syringe and then immediately transferred into the hydrogen-oxygen electrode chamber. Culture broth incubated in the chamber at 30°C produced hydrogen gas proportionally to time after more than 30 min in darkness. The assay of reduced methyl viologen-dependent hydrogen evolution was started by the addition of reduced methyl viologen after bubbling of argon gas into the culture broth to remove hydrogen and oxygen gas, but was not observed in every case.

[b] The concentration of dissolved hydrogen and oxygen recorded in the hydrogen-oxygen electrode system was converted to equilibrating partial pressure (the discrepancy with the gas concentration shown in Fig. 2 was probably due to the disequilibrium between gas phase and culture broth under the conditions shown in Fig. 2).

which was evolving hydrogen and oxygen under the conditions shown in Fig. 2. The culture broth was withdrawn and immediately transferred to the hydrogen-oxygen electrode chamber, but uptake activity in darkness was not observed; hydrogen-evolving activity dependent on endogenous respiration did occur. This means that hydrogen uptake activity by strain N-7363 due to oxy-hydrogen reaction was negligible in comparison to hydrogen evolution in darkness. Nor was light-dependent hydrogen uptake likely to have been active under the aerobic conditions indicated in Fig. 2. Uptake hydrogenase is thought to recover hydrogen gas produced by a side reaction of nitrogenase through anaerobic light-dependent reaction and oxy-hydrogen reaction; the latter is suggested to contribute to the protection of nitrogenase against oxygen (5, 10, 22, 23). In applicational studies, however, addition of carbon monoxide and acetylene (4, 13, 14) and aerobic treatment of darkness (18) were tried to inactivate or repress uptake hydrogenase, which reduces net hydrogen production. The property of this strain described in Table 1 is advantageous for applicational hydrogen evolution since there is no need for artificial prevention of uptake of hydrogen.

Moreover, reduced methyl viologen-dependent hydrogen evolution, which is due to reversible hydrogenase activity according to Houchins (5), was not observed when the samples shown in Table 1 were assayed with the hydrogen-oxygen electrode (data not shown). These facts suggest that the enzyme responsible for hydrogen evolution was nitrogenase only and that hydrogenase may not have been involved in the hydrogen metabolism, at least not under the conditions described in Fig. 2.

Hydrogen accumulation by nitrogen-fixing cyanobacteria has been achieved by Mitsui and co-workers (6, 21) in a closed system with an argon-based atmosphere. However, hydrogen accumulation in the simultaneous presence of nitrogen and oxygen gas has not been reported until now, especially with such a high concentration of oxygen gas as that reported here. A high rate of aerobic hydrogen evolution by *Anabaena* spp. strains CA and 1F (24) has not yet been prolonged in a closed system. *Anabaena* sp. strain N-7363 seems to offer the possibility of hydrogen production under an aerobic or loosely controlled gas atmosphere.

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## LITERATURE CITED

1. **Asada, Y., and S. Kawamura.** 1985. Hydrogen evolving activity among the genus, *Microcystis*, under dark and anaerobic conditions. Rep. Ferment. Res. Inst. **63**:39–54.
2. **Asada, Y., N. Tomizuka, and S. Kawamura.** 1985. Prolonged hydrogen evolution by a cyanobacterium (blue-green alga), *Anabaena* sp. J. Ferment. Technol. **63**:85–90.
3. **Asada, Y., K. Tonomura, and O. Nakayama.** 1979. Hydrogen evolution by an isolated strain of *Anabaena*. J. Ferment. Technol. **57**:280–286.
4. **Daday, A., R. A. Platz, and G. D. Smith.** 1977. Anaerobic and aerobic hydrogen gas formation by the blue-green alga *Anabaena cylindrica*. Appl. Environ. Microbiol. **34**:478–483.
5. **Houchins, J. P.** 1984. The physiology and biochemistry of hydrogen metabolism in cyanobacteria. Biochim. Biophys. Acta **768**:227–255.
6. **Kumazawa, S., and A. Mitsui.** 1981. Characterization and optimization of hydrogen photoproduction by saltwater blue-green alga, *Oscillatoria* sp. Miami BG7. I. Enhancement through limiting the supply of nitrogen nutrients. Int. J. Hydrogen Energy **6**:339–348.
7. **Kumazawa, S., and A. Mitsui.** 1985. Comparative amperometric study of uptake hydrogenase and hydrogen photoproduction activities between heterocystous cyanobacterium *Anabaena cylindrica* B629 and nonheterocystous cyanobacterium *Oscillatoria* sp. strain Miami BG7. Appl. Environ. Microbiol. **50**:287–291.
8. **Lambert, G. R., A. Daday, and G. D. Smith.** 1979. Effects of ammonium ions, oxygen, carbon monoxide, and acetylene on anaerobic and aerobic hydrogen formation by *Anabaena cylindrica* B629. Appl. Environ. Microbiol. **38**:521–529.
9. **Lámbert, G. R., A. Daday, and G. D. Smith.** 1979. Duration of hydrogen formation by *Anabaena cylindrica* B629 in atmospheres of argon, air, and nitrogen. Appl. Environ. Microbiol. **38**:530–536.
10. **Lambert, G. R., and G. D. Smith.** 1981. The hydrogen metabolism of cyanobacteria (blue-green algae). Biol. Rev. **56**:589–660.
11. **Mitsui, A.** 1980. Saltwater based biological solar energy conversion for fuel, chemicals, fertilizer, food and feed, p. 486–491. Proceedings of Bio-Energy '80. World Congress and Exposition. Bio-Energy Council, Washington, D.C.
12. **Miura, Y., H. Yokoyama, K. Kanaoka, S. Saito, K. Iwasa, M. Okazaki, and S. Komemushi.** 1980. Hydrogen evolution by a thermophilic blue-green alga *Mastigocladus laminosus*. Plant Cell Physiol. **21**:149–156.
13. **Miura, Y., H. Yokoyama, M. Kimura, T. Iwamoto, and K. Miyamoto.** 1981. Effect of acetylene and carbon monoxide on hydrogen uptake and evolution by nitrogen-starved cultures of *Mastigocladus laminosus*. Plant Cell Physiol. **22**:1375–1383.
14. **Miura, Y., H. Yokoyama, K. Takahara, and K. Miyamoto.** 1982. Effects of acetylene and carbon monoxide on long-term hydrogen production by *Mastigocladus laminosus*, a thermophilic blue-green alga. J. Ferment. Technol. **60**:411–416.
15. **Miyamoto, K., P. C. Hallenbeck, and J. R., Benemann.** 1979. Solar energy conversion by nitrogen-limited cultures of *Anabaena cylindrica*. J. Ferment. Technol. **57**:287–293.
16. **Miyamoto, K., P. C. Hallenbeck, and J. R. Benemann.** 1979. Nitrogen fixation by thermophilic blue-green algae (cyanobacteria): temperature characteristics and potential use in biophotolysis. Appl. Environ. Microbiol. **37**:454–458.

17. **Miyamoto, K., P. C. Hallenbeck, and J. R. Benemann.** 1979. Hydrogen production by the thermophilic alga *Mastigocladus laminosus*: effects of nitrogen, temperature, and inhibition of photosynthesis. Appl. Environ. Microbiol. **38:**440–446.
18. **Miyamoto, K., K. Takahara-Ishikawa, H. Horie, and Y. Miura.** 1984. Dark-period nitrogen fixation by *Mastigocladus laminosus* and its application to a biophotolysis system. J. Ferment. Technol. **62:**13–18.
19. **Nakayama, O.** 1976. Collection of microalgae in Yamanashi University. Rep. Fac. Eng. Yamanashi Univ. **27:**1–6.
20. **Philips, E. J., and A. Mitsui.** 1983. Role of light intensity and temperature in the regulation of hydrogen photoproduction by the marine cyanobacterium *Oscillatoria* sp. strain Miami BG7. Appl. Environ. Microbiol. **45:**1212–1220.
21. **Reddy, K. J., and A. Mitsui.** 1984. Potential use of aerobic nitrogen fixing unicellular blue-green alga, *Synechococcus* sp. for simultaneous hydrogen and oxygen production from water, p. 179–180. Proceedings of the 7th International Biotechnology Symposium, New Delhi, India.
22. **Stewart, W. D. P.** 1980. Some aspects of structure and function in $N_2$-fixing cyanobacteria. Annu. Rev. Microbiol. **34:**497–536.
23. **Stewart, W. D. P., M. Hawkesford, P. Rowell, and G. A. Codd.** 1982. $H_2$ production from sunlight and $H_2O$—the role of uptake hydrogenase in $N_2$-fixing cyanobacteria, p. 165–169. *In* D. O. Hall and W. Palz (ed.), Photochemical, photoelectrochemical and photobiological processes, series D, vol. 1. D. Reidel Publishing Co., Dordrecht, Holland.
24. **Uebayasi, M., H. Hiura, T. Kakuno, J. Yamashita, N. Tomizuka, and T. Horio.** 1981. Hydrogen-evolving system of photosynthetic bacteria, p. 165. Proceedings of the Annual Meeting of Japanese Plant Physiologists (Sapporo).[In Japanese]
25. **Weissman, J. C., and J. R. Benemann.** 1977. Hydrogen production by nitrogen-starved cultures of *Anabaena cylindrica*. Appl. Environ. Microbiol. **33:**123–131.
26. **Zhang, X., J. B. Haskell, F. R. Tabita, and C. van Baalen.** 1983. Aerobic hydrogen production by the heterocystous cyanobacteria *Anabaena* spp. strains CA and 1F. J. Bacteriol. **156:**1118–1122.