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Aerobic Hydrogen Production by the Heterocystous Cyanobacteria *Anabaena* spp. Strains CA and 1F

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Aerobic photoproduction of H_2 was demonstrated in *Anabaena* spp. strains CA and 1F when cells were growing under nitrogen-fixing conditions. The rates of production, measured either by the hydrogen electrode or in a flow system by gas chromatography, were 10 to 15% of the rate of photosynthetic O_2 evolution or 50 to 80% of the rates of acetylene reduction. Strains CA and 1F differed in several respects. In strain CA, H_2 production was immediately partially sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea, whereas strain 1F was not immediately affected. Strain CA also showed a consistently higher rate of H_2 production than did strain 1F. H_2 production in strain CA was also markedly influenced by the light intensity used for growth, although the growth rates indicated that the light intensities used were essentially saturating.

Heterocystous cyanobacteria (blue-green algae) can evolve hydrogen in a nitrogenase-catalyzed, ATP-dependent reduction of protons. It has been suggested that under aerobic conditions, the hydrogen produced is recycled by an uptake hydrogenase in an energetically useful Knall gas-type reaction (1). Under conditions of nitrogen limitation, e.g., in an argon atmosphere, there is enhanced hydrogen production and minimum hydrogen recycling, with the result that net hydrogen evolution can be found (6, 9, 15). We now wish to report that cultures of heterocystous cyanobacteria growing on N_2 produce hydrogen under aerobic culture conditions at rates 10 to 15% of the rate of photosynthetic O_2 evolution, or 50 to 80% of the rate of acetylene reduction.

MATERIALS AND METHODS

Organisms and culture conditions. Two rapidly growing marine forms of nitrogen-fixing cyanobacteria were used, *Anabaena* spp. strains CA and 1F (3, 12). They were grown on medium ASP-2 (13) free of any added combined nitrogen at 39°C with continuous gassing with 1 ± 0.1% CO_2 in air. With strain CA, the NaCl content of the medium was 5 g/liter; with strain 1F, it was 18 g/liter. The growth baths were illuminated by four fluorescent lamps on each side of the bath, 14 cm from the lamp center to the growth tube center. Two types of lamps were used, F36T12/D/HO and F48T12/CW/1500, and intensities were controlled by screens inserted between the lamps and the bath. Cultures were routinely started from slants every week and grown through several transfers under the desired regime to ensure steady-state conditions. Growth rate and dry weight determinations were done as previously described (2).

Analytical methods. Ethylene was detected and measured by using an Antek model 464 IPC gas chromatograph (Antek Instruments, Inc., Houston, Tex.) equipped with a Chromosorb 104 column (0.33 by 183 cm) (Johns-Manville, Celite Division, Denver, Colo.) held at 50°C.

Hydrogen measurements. Amperometric measurement of hydrogen and oxygen was performed simultaneously, using two no. 5331 electrodes (Yellow Springs Instrument Co., Yellow Springs, Ohio) incorporated into a 2.6-ml water-jacketed electrode chamber (6, 14). The measuring temperature was 39°C. The contents of the electrode chamber were continuously mixed with a small magnetic stirring bar. The electrode signals were detected and amplified by using model 150B microvolt-ammeters (Keithley Instruments, Inc., Cleveland, Ohio) and recorded. Actinic light was provided by a projector with a DAY-DAK 500-W lamp, screened by a no. 34-01-2 hot mirror (Baird-Atomic, Inc., Bedford, Mass.). The lamp was operated at 100 V, and the intensity incident on the electrode chamber was varied by using screens.

The flow system for measuring hydrogen production consisted of a glass tube (22 by 210 mm) with attached 24/40 male joint and a top made of a 24/40 female joint fitted with inlet and outlet tubes, each with a stopcock. Cultures were directly transferred from the growth tubes to the all-glass system, and gas flow at the inlet was begun. The exiting gas was passed through a gas sampling valve with a 1-ml gas sample loop coupled to a model 8500 gas chromatograph (Carle Instruments Inc., Anaheim, Calif.). The hydrogen in the exiting gas stream was measured by periodically diverting the flow from the gas sampling valve through a Spherocarb (Analabs, Inc., North Haven, Conn.) column

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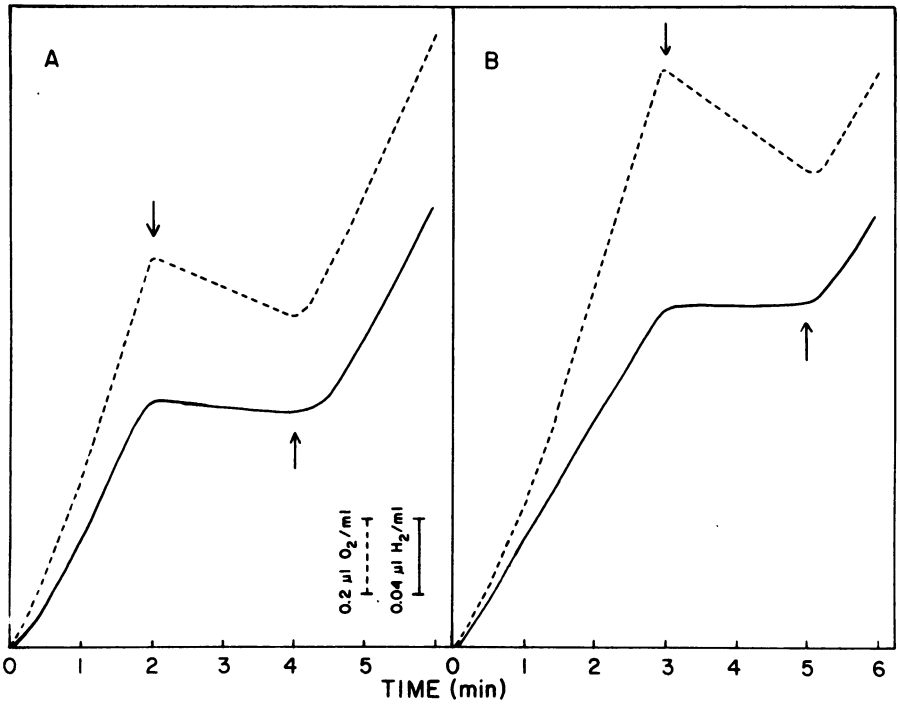

FIG. 1. Tracings, reduced in scale, of simultaneous recordings of H_2 and O_2 production at 39°C by *Anabaena* spp. strains CA (A) and 1F (B). Cultures grown with F48T12/CW/1500 lamps were transferred directly from the growth tube to the electrode chamber and gassed for 2 min with 1% CO_2 in air. This eliminated the background level of H_2 always present in the cultures. The intensity incident upon the front of the electrode chamber was 1,200 $\mu E/m^2$ per s. The cell densities were 0.13 mg (dry weight) per ml for strain CA and 0.16 mg (dry weight) per ml for strain 1F. Arrows indicate light on (↑) or light off (↓).

(0.33 by 213 cm) held at 37°C. Hydrogen was identified by retention time and quantitated by peak height. Mass spectrometry was also used to verify qualitatively that the gas samples from the cultures contained hydrogen.

RESULTS AND DISCUSSION

Figure 1 shows typical rates of H_2 production when cells were transferred from the growth bath to the electrode chamber and measurements were begun within several minutes. The curves represent for strain CA an aerobic H_2 production rate of 32 µl and an O_2 evolution rate of 250 µl; for strain 1F the rates were 24 µl of H_2 and 210 µl of O_2, all per milligram (dry weight) per hour. The activity was strictly light dependent. In the case of strain CA, the immediate small dark H_2 uptake was taken to indicate a low level of uptake hydrogenase activity, whereas with strain 1F, there was even less indication of uptake hydrogenase activity. Strains CA and 1F behaved differently upon addition of DCMU to the electrode chamber. Photosynthetic O_2 evolution ceased, but H_2 production continued in both organisms, albeit at a lower rate in strain CA than in strain 1F (Fig. 2). In general, it is believed that DCMU will not immediately block H_2 production caused by nitrogenase (6); however, different effects were seen in *Anabaena cylindrica* depending upon the prior history of the cells (8). The differences in DCMU sensitivity in strains CA and 1F may simply reflect differences in the reductant pool(s), or they may indicate that some part of the H_2 production in CA is closely linked to photosynthesis. The argument can be made that if H_2 can indeed be formed directly via photosynthesis, it will be evident only in nitrogen-fixing cyanobacteria grown on N_2 wherein the requisite highly active hydrogenase(s) is coordinately induced.

The results with strains CA and 1F, using the hydrogen electrode, were completely verified by using closed growth tubes under normal aerobic culture conditions (Fig. 3). Strain CA again behaved differently from strain 1F, giving higher rates of H_2 production. The H_2 production was, within limits, independent of the flow rate of gas through the culture or of cell density. There was little effect on H_2 production when the gas phase was shifted from 1% CO_2 in air to 1% CO_2 in N_2.

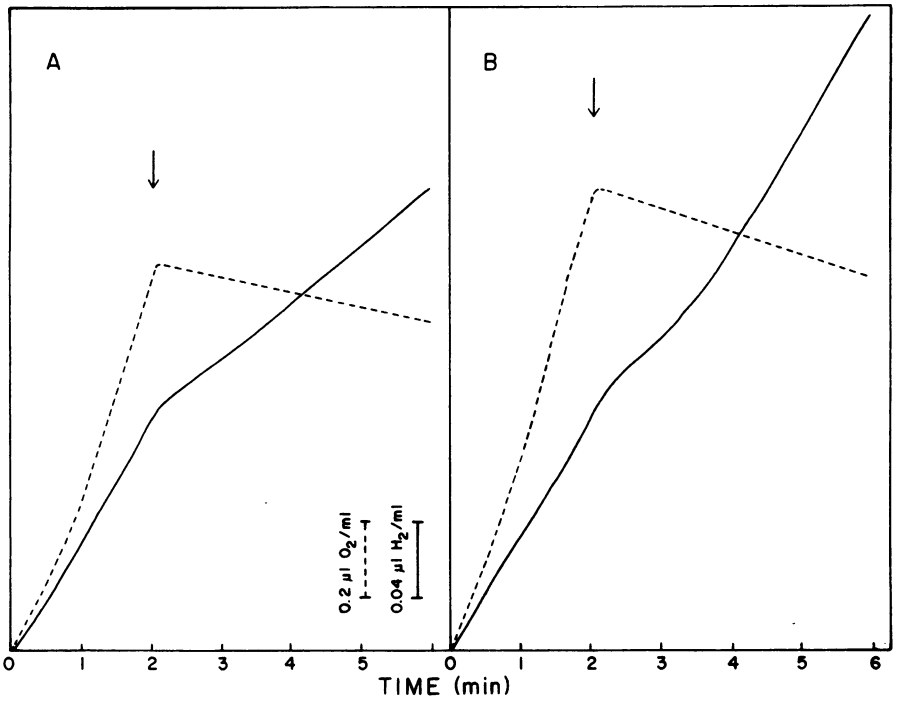

FIG. 2. Effect of DCMU on the photoproduction of H_2 and on O_2 evolution by *Anabaena* spp. strains CA (A) and 1F (B). Growth conditions, cell densities, and measurng conditions were as described in the legend to Fig. 1. The DCMU was dissolved in 95% ethanol to a final concentration of 10^{-5} M. The addition of DCMU is indicated by arrows.

With a shift to 1% CO_2 in argon, there was an increase in H_2 production to the very high rate of 80 µl of H_2 per mg (dry weight) per h, approximately 30% of the measured rate of photosynthetic O_2 evolution.

H_2 production was higher in the flow system than on the H_2 electrode, perhaps due to the unavoidable buildup of H_2 in the electrode chamber or to some slight toxicity of materials in contact with the cells. In any event, the simple flow system minimizes disturbances to the cells and yields values believed to be most nearly representative of the steady-state growth condition. Although these aerobic H_2 production values, ca. 40 µl of H_2 per mg (dry weight) per h in strain CA, are the highest ever achieved with nitrogen-fixing cyanobacteria, still higher rates of 130 µl of H_2 per mg (dry weight) per h have been recorded in the photosynthetic bacterium *Rhodopseudomonas capsulata* (4). However, in contrast to the aerobic cyanobacterial photoproduction of H_2, in which H_2O is the ultimate electron donor, the photosynthetic bacteria produce H_2 from organic compounds by an anaerobic light-dependent process. The bacterial system is thus energetically and experimentally less favorable than the cyanobacterial system.

For the present, we have little understanding of the underlying physiological or biochemical characteristics in cells which predispose towards aerobic hydrogen production. We leave aside any arguments based upon the synthesis and regulation of uptake hydrogenases in strains CA and 1F as we have, as yet, little information on this point. There was a relationship between the light intensities below 180 µE/m^2 per s and hydrogen production (Table 1), with little change in growth rate. In strain CA especially, the higher the light intensity, the higher the rate of aerobic hydrogen production. A possibly related observation is an increased H_2 production in "photobleached" cells of *A. cylindrica*, although after the photobleaching treatment, the cells stopped growing (7). A possible explanation for the high aerobic H_2 production in strains CA and 1F is provided by the general observations on oxygen uptake by light-generated reductant (5, 10, 11), presumably a protective mechanism when CO_2 supply is restricted in a cell. In nitrogen-fixing cyanobacteria wherein the requisite H_2 handling machinery is necessarily present, excess light-generated electron flow may be shunted to hydrogen production in place of being controlled by a "back-reaction" with

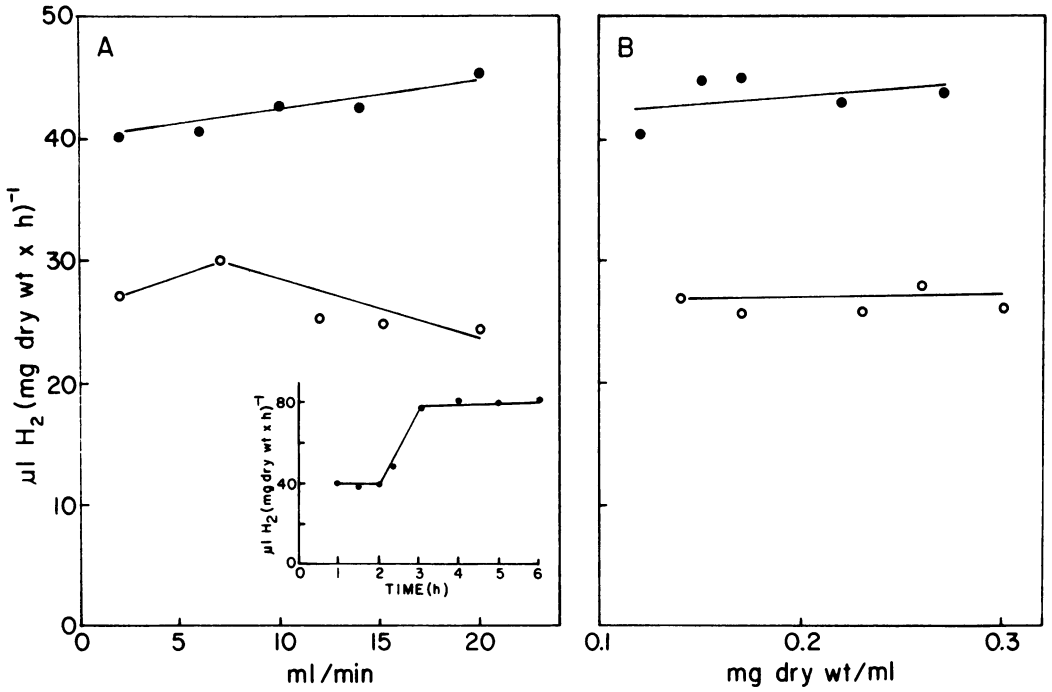

FIG. 3. H_2 production by *Anabaena* spp. strains CA and 1F in sealed, all-glass growth tubes as a function of flow rate of 1% CO_2 in air through the culture and as a function of culture density. The light intensity for growth and measurement of H_2 production was 450 $\mu E/m^2$ per s (F48T12/CW/1500 lamps). The cell density for the flow rate experiment (A) was 0.2 mg (dry weight) per ml. The flow rate for the cell density experiment (B) was 6 ml/min. Solid circles indicate strain CA; open circles indicate strain 1F. The inset figure in panel A shows the effect on H_2 production in strain CA of shifting the gassing mixture from 1% CO_2 in air to 1% CO_2 in argon at 2 h. There was little change in the rate of H_2 production when the gas mixture was shifted from 1% CO_2 in air to 1% CO_2 in N_2 (data not shown).

O_2. If this is true, it suggests that it may be most fruitful to search via enrichment culture or mutation for strains of nitrogen-fixing cyanobacteria that are not impaired in photosynthetic electron flow but are limited in oxidant utilization, either CO_2 or N_2.

TABLE 1. Effect of light intensity on growth rate and aerobic H_2 production and O_2 evolution in *Anabaena* spp. strains CA and IF[a]

Light intensity		Generation time (h)		H_2 production (μl/mg [dry wt] per h)		O_2 evolution (μl/mg [dry wt] per h)	
No. of screens	$\mu E/m^2$ per s	CA	IF	CA	IF	CA	IF
0	180	4.5	4.8	19	21	259	260
1	75	4.7	4.8	9	20	254	256
2	35	5.0	5.5	3	14	231	254
3	20	5.6	5.7	2	14	230	260

[a] Conditions for electrode measurements of H_2 and O_2 were the same as in Fig. 1. The cultures were grown at 39°C and illuminated by F36T12/D/HO fluorescent lamps.

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LITERATURE CITED

1. **Bothe, H., J. Tennigkeit, and G. Eisbrenner.** 1977. The utilization of molecular hydrogen by the blue-green alga, *Anabaena cylindrica*. Arch. Microbiol. **114**:43–49.
2. **Bottomley, P. J., and C. Van Baalen.** 1978. Characteristics of heterotrophic growth in the blue-green alga *Nostoc* sp. strain Mac. J. Gen. Microbiol. **107**:309–318.
3. **Gotto, J. W., F. R. Tabita, and C. Van Baalen.** 1979. Isolation and characterization of rapidly-growing, marine, nitrogen-fixing strains of blue-green algae. Arch. Microbiol. **121**:155–159.
4. **Hillmer, P., and H. Gest.** 1977. H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: H_2 production by growing cultures. J. Bacteriol. **129**:724–731.
5. **Hoch, G., O. V. H. Owens, and B. Kok.** 1963. Photosynthesis and respiration. Arch. Biochem. Biophys. **101**:171–180.
6. **Jones, L. W., and N. I. Bishop.** 1976. Simultaneous measurement of oxygen and hydrogen exchange from the blue-green alga, *Anabaena*. Plant Physiol. **57**:659–665.

7. **Laczko, I., and K. Barabas.** 1981. Hydrogen evolution by photobleached *Anabaena cylindrica*. Planta **153**:312–316.
8. **Lex, M., and W. P. D. Stewart.** 1973. Algal nitrogenase, reductant pools and photosystem I activity. Biochim. Biophys. Acta **292**:436–443.
9. **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.
10. **Radmer, R. J., and B. Kok.** 1976. Photoreduction of O_2 primes and replaces CO_2 assimilation. Plant Physiol. **58**:336–340.
11. **Radmer, R. J., and O. Ollinger.** 1980. Light-driven uptake of oxygen, carbon dioxide, and bicarbonate by the green alga, *Scenedesmus*. Plant Physiol. **65**:723–729.
12. **Stacey, G., C. Van Baalen, and F. R. Tabita.** 1977. Isolation and characterization of a marine *Anabaena* sp. capable of rapid growth on molecular nitrogen. Arch. Microbiol. **114**:197–201.
13. **Van Baalen, C.** 1962. Studies on marine blue-green algae. Bot. Mar. **4**:197–201.
14. **Wang, R., F. P. Healey, and J. Myers.** 1971. Amperometric measurement of hydrogen evolution in *Chlamydomonas*. Plant Physiol. **48**:108–110.
15. **Weissman, J. C., and J. R. Benemann.** 1977. Hydrogen production by nitrogen-starved cultures of *Anabaena cylindrica*. Appl. Environ. Microbiol. **33**:123–131.