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Physiological Conditions for Nitrogen Fixation in a Unicellular Marine Cyanobacterium, *Synechococcus* sp. Strain SF1†

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A marine, unicellular, nitrogen-fixing cyanobacterium was isolated from the blades of a brown alga, *Sargassum fluitans*. This unicellular cyanobacterium, identified as *Synechococcus* sp. strain SF1, is capable of photoautotrophic growth with bicarbonate as the sole carbon source and dinitrogen as the sole nitrogen source. Among the organic carbon compounds tested, glucose and sucrose supported growth. Of the nitrogen compounds tested, with bicarbonate serving as the carbon source, both ammonia and nitrate produced the highest growth rates. Most amino acids failed to support growth when present as sole sources of nitrogen. Nitrogenase activity in *Synechococcus* sp. strain SF1 was induced after depletion of ammonia from the medium. This activity required the photosynthetic utilization of bicarbonate, but pyruvate and hydrogen gas were also effective sources of reductant for nitrogenase activity. Glucose, fructose, and sucrose also supported nitrogenase activity but to a lesser extent. Optimum light intensity for nitrogenase activity was found to be 70 μE/m² per s, while the optimum oxygen concentration in the gas phase for nitrogenase activity was about 1%. A hydrogenase activity was coinduced with nitrogenase activity. It is proposed that this light- and oxygen-insensitive hydrogenase functions in recycling the hydrogen produced by nitrogenase under microaerobic conditions.

The enzyme nitrogenase catalyzes the reduction of dinitrogen to ammonia. Due to the extreme sensitivity of this enzyme to oxygen, nitrogen fixation by microorganisms occurs when oxygen can be prevented from inactivating the nitrogenase. Heterotrophic bacteria require an oxygen-limited environment, which permits nitrogen fixation to occur when the respiration rate of the cells equals or exceeds the rate of diffusion of oxygen to the cell surface (25). In this regard, the nitrogen-fixing cyanobacteria present a special problem during oxygenic photosynthesis, when the amount of oxygen evolved in the light exceeds noninhibitory levels for the nitrogenase enzyme. Several filamentous cyanobacteria produce specialized cells, heterocysts, which protect the nitrogenase from oxygen (24). However, aerobic nitrogen fixation also occurs in nonheterocystous, filamentous organisms such as *Oscillatoria* spp. and unicellular cyanobacteria such as *Gloeothece* spp. (4, 6, 17, 21). This second group of cyanobacteria is generally found in large clumps or bundles of filaments, and this growth pattern may limit the rate of oxygen diffusion into the interior parts of the cell cluster. Rippka and Waterbury (18) reported that certain *Synechococcus* species, growing as solitary, unicellular organisms, produced nitrogenase activity only under anaerobic conditions. Huang and Chow (5) and Mitsui and co-workers (11, 14) have also isolated and described a *Synechococcus* species which produced nitrogenase activity under aerobic conditions. Nitrogenase activity in both *Gloeothece* and *Synechococcus* was supported by respiration and by low levels of light, supplying most likely only ATP for this process (10, 12). However, the presence and role of hydrogenase as a supporting enzyme complex in these systems have not been reported.

We isolated a unicellular, nitrogen-fixing cyanobacterium from the blades of *Sargassum fluitans* and classified it as *Synechococcus* sp. (strain SF1). This organism is capable of growth with dinitrogen as the sole source of nitrogen, under limiting oxygen concentrations. This strain also produced a light-insensitive hydrogenase activity, which was coinduced with nitrogenase. The physiological conditions under which nitrogenase activity is expressed in this *Synechococcus* species are presented in this communication.

MATERIALS AND METHODS

Materials. Biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Inorganic and organic chemicals were obtained from Fisher Scientific Co. (Orlando, Fla.) and were of analytical grade.

Media and growth conditions. *Synechococcus* sp. was grown in ASN III medium (16), modified to include 15 mM TES [*N*-tris(hydroxymethyl)methyl-2-amino ethanesulfonic acid] as buffer (pH 7.15). Sodium bicarbonate was included as a carbon source, and either ammonium chloride or potassium nitrate (5 mM) served as a source of nitrogen. When CO_2 was used as the carbon source, air enriched with CO_2 (0.8%) was sparged through the cultures (usually 20 ml of medium in a 25 by 200 mm tube) at a flow rate of 12 ml/min. Continuous light from cold-fluorescence tubes (40 W) provided a light intensity of 100 μE/m² per s for growth. Bacterial contamination of the culture was regularly checked by plating samples of the culture on modified ASN III medium supplemented with sucrose (0.3%) and Casamino Acids (0.8%). For growth experiments testing the utilization of various carbon and nitrogen compounds as C or N sources, 20-ml screw-cap culture tubes were used with 10 ml of medium. In experiments evaluating the effect of carbon sources on growth, NH_4^+ (5 mM) served as the N source. When checking the effect of nitrogen compounds on growth, the culture medium was supplemented with bicarbonate (10 mM) as the carbon source, under a gas phase of argon, to prevent dinitrogen fixation. All experiments were carried out in duplicate, and each growth series was repeated several times. The results represent the mean of two values for a single experiment.

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TABLE 1. Growth characteristics of *Synechococcus* sp. strain SF1 with different carbon sources[a]

Carbon source	Cell yield (OD_{750})		Concn (μg/ml)			
			Protein		Chlorophyll	
	Without DCMU	With DCMU	Without DCMU	With DCMU	Without DCMU	With DCMU
No addition	0.10	UD[b]	3	UD	<0.5	UD
Bicarbonate	2.00	UD	90	UD	5.8	UD
Glucose	1.44	0.23	64	28	4.0	0.4
Fructose	0.60	0.16	40	UD	1.9	UD
Sucrose	0.96	0.16	64	28	2.8	0.6
Mannitol	0.26	0.12	18	15	0.5	0.2
Galactose	0.60	0.14	60	15	1.5	0.3
Acetate	0.42	0.20	30	15	1.0	UD
Succinate	1.00	0.16	35	15	3.2	0.3

[a] All carbon sources were present at an initial concentration of 5 mM, except bicarbonate (10 mM). The nitrogen source was ammonia at an initial concentration of 5 mM. DCMU was added at 20 μM. Cell yield was determined after 15 days of growth at 25°C. The OD_{750} of the culture immediately after inoculation was 0.05.

[b] UD, Undetectable by the standard methods used.

Analytical methods. Nitrogenase and hydrogenase activities were determined as described previously (19, 20). The general protocol for measuring hydrogenase activity by the tritium exchange assay is described below. For the exchange of $^3H^+$ from $^3H_2$ with H^+ from water, 10 to 50 μl of tritium gas (11.2 mCi/mmol; New England Nuclear) were injected into assay vials equilibrated with He and other gases as needed. After specific time intervals, 50 to 100 μl of the aqueous phase was withdrawn and diluted threefold with water. Gaseous tritium was removed by vigorous mixing. The tritiated water content of the samples was determined in a liquid scintillation counter after addition of 2.5 ml of scintillation fluid. After subtracting the counts from an aqueous control without cells, the specific activity of hydrogenase was calculated. The variation between duplicate samples was generally <5%.

The ammonia concentration was monitored with Nessler reagent (20). Cell growth was measured as optical density at 750 nm (OD_{750}). Protein and chlorophyll contents of the cells were determined by the methods of Lowry et al. (8) and MacKinney (9), respectively. Photosynthetic O_2 evolution was measured with a Clark-type electrode in a water-cooled cell at 25°C, illuminated with red light provided by a filter of 2-mm thickness and a transmission of 10% at 610 nm. Spectral characteristics of the organism were determined with an SLM-Aminco dual-wavelength spectrophotometer.

RESULTS

Isolation and growth. *Synechococcus* sp. strain SF1 was isolated from a pelagic brown alga, *S. fluitans*, collected from the Atlantic Ocean within the reef east of Big Pine Key, Florida. Blade segments were thoroughly washed with sea water and embedded in modified ASN III soft agar (0.6%) medium lacking combined nitrogen, and with ambient CO_2 as the carbon source. Plates were incubated in the light at room temperature (25°C). Repeated transfers of cells on bicarbonate medium produced single colonies of the unicellular cyanobacterium free from contaminating organisms. For growth experiments, cells were transferred to 20-ml screw-cap tubes, holding 12 ml of ASN III medium supplemented with bicarbonate (10 mM) and ammonia (5 mM). Alternatively, cells were grown in batch cultures in 125-ml Erlenmeyer flasks, with 50 ml of inorganic ASN III medium. Ambient CO_2 was the carbon source, and nitrate (5 mM) served as the N source. *Synechococcus* cells appeared oblong to disk shaped and varied in size from 4 to 6 μm in length and 2.5 to 3 μm in diameter. Chains of attached cells could be found in undisturbed cultures, and the frequent occurrence of twin cells was taken as an indication that this organism divided in a single plane. Based on Stanier's classification of cyanobacteria (16, 22), this isolate, designated strain SF1, was identified as a *Synechococcus* sp.

Effect of carbon compounds on growth. In growth experiments, strain SF1 grew well with bicarbonate as a carbon source and ammonia as a nitrogen source (Table 1), with a generation time of 20 h, at 25°C. Several organic carbon compounds increased the cell yield over the control. The control culture after 15 days of growth showed an increase in OD which was barely above the cell density of the initial inoculum (OD_{750} of 0.1 versus 0.05). Among the organic carbon compounds tested, glucose, sucrose, and succinate provided the highest cell yields, measured as both OD and protein. Galactose was equally efficient in cell protein production, but the OD did not increase at the same rate as in glucose-grown cultures. Mannitol, acetate, and citrate, supplied as Na^+ salts, were poor substrates for growth, determined as either cell yield, protein, or chlorophyll. Pyruvate did not stimulate growth of the organism under aerobic conditions (not shown), but in a closed system under a dinitrogen atmosphere and in the absence of ammonium ions, growth in the presence of pyruvate as the C source approached that of the bicarbonate-ammonia sample. These results and the requirement of a closed system for growth in a pyruvate medium suggest that the organic compounds were metabolized to produce CO_2 and the CO_2 was reassimilated, leading to the observed growth.

If the observed cell yield in the presence of fixed carbon was due to complete decomposition of these organic carbon compounds to CO_2 and its refixation, the addition of 20 μM DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], an inhibitor of photosynthetic electron transport between the two photosystems and thus an inhibitor of CO_2 fixation, to the growth medium ought to completely abolish all growth. DCMU inhibited growth mediated by CO_2 or fructose, measured as increase in cell protein (Table 1). All other compounds tested, however, supported growth of strain SF1 to various degrees, but the cell yield never reached the values observed without DCMU. These results show that *Synechococcus* sp. strain SF1 can use preformed organic compounds, especially glucose, to support growth directly, albeit to a lesser extent.

During growth in the presence of organic carbon compounds, the cells produced very little extracellular polysaccharide. However, large amounts of capsular polysaccharide could be easily detected in N-limited, older cultures which had continuously been sparged with CO_2-enriched air. Furthermore, growth in a medium of bicarbonate-ammonia (10 mM) did not lead to any detectable slime production.

Effect of nitrogen compounds on growth. Ammonia, nitrate, and N_2 were the preferred nitrogen sources among the compounds tested (Table 2). Good growth in asparagine medium could have resulted from decomposition of the compound to yield ammonia. Glutamate, glutamine, aspartate, glycine, and other amino acids failed to support growth of this organism. Glutamate and glutamine, as well as serine, leucine, lysine, and tryptophan, led to cell yields lower than the control, indicating inhibition of growth. By lowering the concentration of these amino acids to 1 mM or less to

overcome possible inhibitory effects, the cell yield was not substantially increased.

Synechococcus sp. strain SF1 was capable of growing with dinitrogen as the sole nitrogen source (Table 2). In the presence of N_2 and bicarbonate, the culture grew with a generation time of about 38 h, which is about twice the generation time of a similar ammonia-grown culture (20 h, Table 3). The protein content of the N_2-HCO_3^- culture was about 65% of that of the control cultures (air or N_2-HCO_3^- and NH_4^+, 71 versus 110 μg/ml). Although dinitrogen-dependent growth was detected under aerobic conditions, the growth rate (49-h generation time) and cell yield (35 μg of protein per ml) of these cultures were lower than those of a microaerobic culture that had been started under a dinitrogen atmosphere. The high oxygen content (21%) of ambient air obviously reduced the growth rate. Continuous sparging of the culture with air also eliminated N_2-dependent growth, while shaking at 50 rpm only decreased the growth rate. The inhibitory effect of oxygen on growth was only seen when dinitrogen served as the sole N source and not with an NH_4^+ culture.

Induction of nitrogenase activity. When a *Synechococcus* culture was transferred to fresh medium containing limiting ammonium ion, nitrogenase activity developed only after the ammonia was exhausted from the medium (Fig. 1). Nitrogenase activity in the air-CO_2-sparged culture increased linearly with time, and the maximum activity was observed when the culture reached the stationary phase of growth. Concomitant with this increase in nitrogenase activity was an increase in hydrogenase activity. Prior to the development of nitrogenase activity, the activity of hydrogenase in the light was near zero from day 1 to day 4. However, the culture doubled once during the period in which nitrogenase and hydrogenase activities were observed.

In a separate experiment, the effect of air versus nitrogen gas on the induction of nitrogenase and hydrogenase activities was determined (Fig. 2). Cells from an ammonia-depleted culture (Fig. 1) were removed from the culture vessel, washed free of residual NH_4^+, and suspended in fresh medium. This culture was incubated in closed containers with bicarbonate as the carbon source, under a gas phase of either dinitrogen or air. Under these experimental conditions, nitrogenase activity could be detected after about 16 h, and this activity increased exponentially. The maximum nitrogenase activity produced by the culture under air (static) was higher than that in the air-sparged culture used in the previous experiment (with 4 to 6 versus 1 μmol of ethylene produced per h per mg of chlorophyll; Fig. 1). The culture with dinitrogen as the starting gas phase, however, produced three times more nitrogenase activity than the static air culture. The hydrogenase activity of the sample that was kept in air was very low and stayed constant. However, the hydrogenase activity of the sample with N_2 in the gas phase increased linearly until it reached a maximum activity of 3.6 μmol/h per mg of chlorophyll.

Enhancement of nitrogenase activity in whole cells by externally added carbon or hydrogen gas. The synthesis and activity of nitrogenase require both reducing power and energy. In strain SF1, energy is supplied by light. Since the organism is capable of utilizing organic carbon compounds, the role that these compounds play in the production of nitrogenase activity was tested (Table 4). For these experiments, the rate of nitrogenase activity produced by an NH_4^+-depleted culture was determined between 28 and 40 h after ammonia was removed from the medium. This time period was chosen because the activity of nitrogenase in-

TABLE 2. Effect of various nitrogen sources on growth of *Synechococcus* sp. strain SF1[a]

Nitrogen source	Cell yield (OD_{750})
None	0.35
Ammonium ion	1.36
Nitrate	1.20
Glutamate	0.05
Glutamine	0.13
Aspartate	0.24
Asparagine	0.93
Glycine	0.30
N_2	1.03

[a] The inoculum for this experiment was grown in ASN III medium with bicarbonate (10 mM) as the carbon source and NH_4^+ (5 mM) as the nitrogen source. These cells were washed free of ammonia and used to inoculate fresh medium with the various nitrogen sources at an initial concentration of 5 mM. Bicarbonate at 10 mM served as the carbon source. A 20-ml portion of this culture was transferred to 70-ml bottles, and the gas phase was replaced with Ar. Cultures were incubated at 30°C under continuous light (70 μE/m^2 per s). The initial OD_{750} was 0.04, and the final OD was determined after 10 days.

TABLE 3. Effect of oxygen on growth of *Synechococcus* sp. strain SF1 with dinitrogen as the nitrogen source[a]

Gas phase	Carbon source	Nitrogen source	Generation time (h)	Cell yield (μg of protein/ml)
Air or N_2	HCO_3^-	NH_4^+	20	110
Air	CO_2	NH_4^+	40	60
Air	CO_2	N_2	No growth	0
Air	CO_2[b]	N_2	No growth	18
Air	HCO_3^-	N_2	49	35
N_2	HCO_3^-	N_2	38	71

[a] Anaerobic cultures were grown in 10 ml of medium in 70-ml stoppered bottles under an N_2 atmosphere. Aerobic cultures were grown in 125-ml flasks with gentle mixing (100 rpm). When bicarbonate and ammonia were supplied, their concentrations were 10 and 5 mM, respectively. The CO_2 concentration was 0.8% in air unless indicated otherwise. The OD_{750} of the initial inoculum for all samples was 0.1; the final OD_{750} of the HCO_3^--NH_4^+ sample was 1.32. For other conditions, see the text.

[b] Ambient concentration.

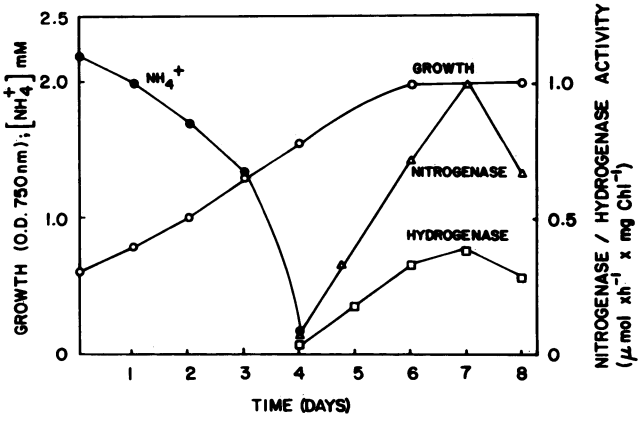

FIG. 1. Induction of nitrogenase and hydrogenase activities in *Synechococcus* sp. strain SF1. A culture was suspended in 5 mM bicarbonate and in limiting ammonia (1.5 mM) and placed in the light, in air. At the times indicated, samples were taken to determine growth, ammonia content, and nitrogenase and hydrogenase activities. Chl, Chlorophyll.

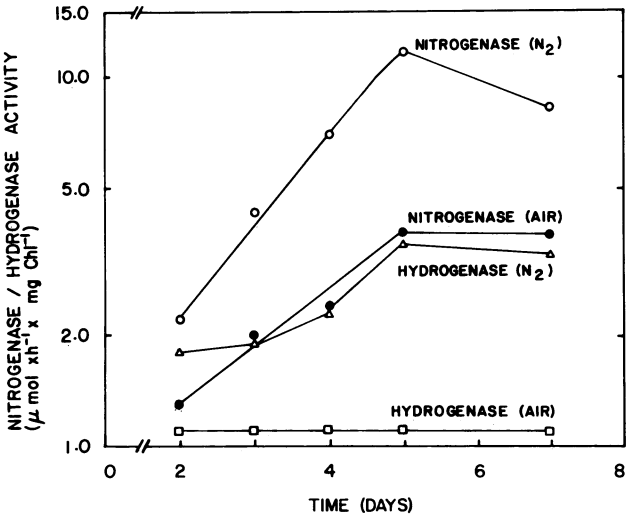

FIG. 2. Effect of gas phase, air or nitrogen, on induction of nitrogenase and hydrogenase activities by *Synechococcus* sp. strain SF1. An ammonia-CO_2 culture was washed free of spent medium and transferred to 70-ml stoppered vials in 20 ml of fixed-N-free medium supplemented with 10 mM bicarbonate. At the times indicated, 1-ml samples were transferred to anaerobic assay vials, and nitrogenase and hydrogenase activities were determined for a 4-h period. Chl, Chlorophyll.

creased exponentially between 16 and 120 h after ammonia removal (Fig. 2). The control with no addition produced low levels of activity (1 μmol of ethylene per h per mg of chlorophyll), probably by utilizing endogenous reserves. This low activity could be completely abolished if the cells were starved under continuous light before the assay. Both bicarbonate and pyruvate produced an increase in nitrogenase activity over the control (by 3.7-fold and 3.2-fold, respectively), which was only surpassed by the sample with hydrogen (increase by 4.7-fold). Glucose, fructose, and sucrose did enhance nitrogenase activity, although at a lower level. In the presence of DCMU, which inhibits photosystem II but increases oxygen uptake in the light (not shown), the nitrogenase activity of all samples was higher than that of the control. Addition of DCMU reduced the bicarbonate-mediated enhancement of nitrogenase activity to about 25% of the control. On the other hand, the presence of DCMU increased the effect of pyruvate and H_2. Supplementation of the H_2 culture with bicarbonate had no effect. The enhancement of nitrogenase activity by pyruvate in the presence of DCMU suggests that the reductant generated from pyruvate or hydrogen oxidation is directly coupled to nitrogenase and not dependent on fixation of CO_2 to compounds which may act as carriers of reductant for nitrogenase activity.

Effect of oxygen on nitrogenase activity. The results presented in Tables 3 and 4 and Fig. 2 suggest that the production of nitrogenase activity by strain SF1 was sensitive to oxygen. To determine the extent of this sensitivity, the effect of O_2 on nitrogenase activity was measured with whole cells. Nitrogenase activity was assayed under low light (40 μE/m^2 per s), which was optimal (Fig. 3). At this light intensity, the activity of nitrogenase was enhanced by up to 1% (vol/vol) oxygen (2.43 μmol/h per mg of chlorophyll). However, between 1 and 10% oxygen, nitrogenase activity decreased exponentially and was undetectable at 10% oxygen. Although nitrogenase activity in the dark was undetectable, hydrogenase activity in the dark was at a maximum under anaerobic conditions (1.72 μmol/mg of chlorophyll per h).

Effect of light intensity on nitrogenase and hydrogenase activities. The experimental results presented in Fig. 2 show that the nitrogenase and hydrogenase activities were sensitive to oxygen. Since photosynthesis in cyanobacteria generates oxygen and light is a critical factor for growth of cyanobacteria (23), the optimum light intensity for nitrogen fixation was also determined. For these experiments, cells grown under aerobic conditions with bicarbonate as the carbon source were used. The evolution of O_2 by strain SF1 followed a typical photosynthetic light saturation pattern, with O_2 uptake in low light (below 30 μE/m^2 per s) followed by an exponential increase in the rate of O_2 evolution with increasing light intensity, until light saturation was achieved (Fig. 3). The maximum rate of O_2 evolution occurred near 1,200 μE/m^2 per s, and the compensation point for photosynthetic O_2 evolution was at about 30 μE/m^2 per s. Nitrogenase activity was detected at light intensities as low as 3 μE/m^2 per s, although no activity was observed in the dark. Nitrogenase activity rose with increasing light flux until inhibition started at about 80 μE/m^2 per s. At higher light intensities, the activity was progressively reduced. The optimum conditions for nitrogen fixation were attained at about 10% of the light intensity required for the maximum photosynthetic rate of oxygen evolution.

Hydrogen uptake activity (HUP) of hydrogenase is associated with electron transport coupled to dinitrogen fixation. It was measured under identical conditions used for nitrogenase activity, with the same cell material. This hydrogenase activity was not influenced by light intensity up to about 65 μE/m^2 per s. Even at a light intensity exceeding the light saturation of oxygen evolution (1,000 μE/m^2 per s), this culture maintained about 70% of its maximum hydrogenase activity and about 45% of its nitrogenase activity.

These results show that the air-grown cultures maintained high rates of nitrogenase and hydrogenase activities at light intensities near the photosynthetic compensation point.

DISCUSSION

Studies on unicellular, nitrogen-fixing cyanobacteria have focused mainly on organisms that were capable of protecting

TABLE 4. Enhancement of nitrogenase activity in whole cells of *Synechococcus* sp. strain SF1 by added hydrogen or carbon compounds[a]

Addition	Nitrogenase activity (μmol of ethylene/h per mg of chlorophyll)	
	Without DCMU	With DCMU
None	1.0	1.0
HCO_3^-	3.7	1.7
Glucose	2.2	2.6
Fructose	1.9	2.8
Sucrose	2.4	2.3
Mannitol	0.9	1.5
Citrate	0.8	1.3
Pyruvate	3.2	5.6
H_2	4.6	6.2
H_2 + HCO_3^-	4.7	7.0

[a] An NH_4^+-bicarbonate-grown culture was washed and suspended in ASN III medium and incubated under Ar with 10% C_2H_2 in the light. Additions were made after 12 h, when nitrogenase activity was detectable. Nitrogenase activity was determined between 28 and 40 h, when the control attained the maximum level of activity. The initial concentration of all compounds was 5 mM, except for pyruvate and HCO_3^-, which were 10 mM each, and DCMU was added at 10 μM.

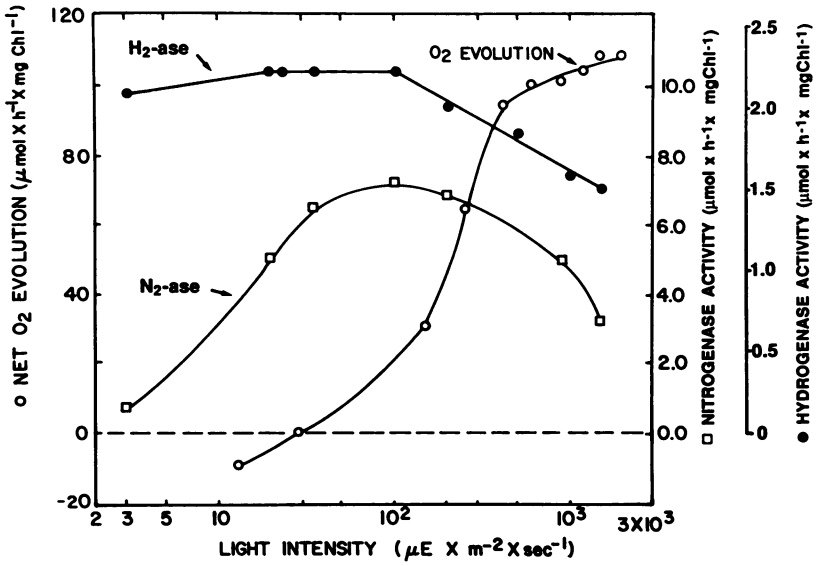

FIG. 3. Effect of light intensity on net oxygen production, nitrogenase activity, and hydrogenase activity of *Synechococcus* sp. strain SF1. A dinitrogen-CO_2-grown culture, induced for nitrogenase activity as described for Fig. 2, was used in this experiment. Hydrogenase activity was determined in a helium atmosphere. Chl, Chlorophyll.

nitrogenase from oxygen in the environment as well as from photosynthetically produced oxygen (6, 24). *Gloeothece* spp. produce a slime layer to exclude oxygen from the nitrogenase (2, 3). Although dinitrogen fixation in *Gloeothece* spp. occurs under continuous illumination, respiration is the major source of energy for dinitrogen fixation (10), and oxygen still inhibits nitrogenase activity (1). A *Synechococcus* sp. with very low levels of extracellular polysaccharide has been reported to produce nitrogenase only under anaerobic conditions (18). Although the nitrogenase activity could be detected readily in this organism, growth at the expense of dinitrogen was not reported. More recently, Mitsui and co-workers isolated and described several strains of marine *Synechococcus* spp. which were capable of photosynthetic growth in combined, nitrogen-free medium (11, 14). Using synchronously divided cultures of strain Miami BG43511, these investigators found that nitrogen fixation occurred only at specific stages in the cell cycle (11). The role of hydrogenase enzyme in relation to nitrogenase in these isolates is totally unknown.

The *Synechococcus* sp. strain SF1 we have isolated and described is also capable of aerobic growth with dinitrogen as the nitrogen source. However, special precautions were needed during growth to protect the nitrogenase from oxygen inactivation. Dinitrogen-dependent growth could be initiated from a very low inoculum but only under anaerobic conditions, whereas air-CO_2-sparged cultures failed to grow with N_2 as the N source (Table 3). However, if growth was initiated with limiting amounts of NH_4^+, nitrogenase induction in these air-CO_2 cultures could be detected readily. This difference in the appearance of nitrogenase activity could be attributed to higher cell densities prevalent in the limiting NH_4^+ culture when the nitrogen source was exhausted. The lowest cell density required for the production of detectable nitrogenase activity was at an OD_{750} of ca. 1. This higher cell density obviously decreased the effective light intensity and the evolution of oxygen resulting from it. Increasing the light intensity or decreasing the cell density to saturate photosynthetic rates reduced nitrogenase activity, probably a consequence of enhanced oxygen evolution. The optimum light intensity for nitrogen fixation was near the compensation point for oxygen (Fig. 3).

In the absence of nitrogen fixation, *Synechococcus* sp. strain SF1 grew well with bicarbonate as a carbon source with the shortest measured generation time of about 20 h, at 25°C. However, the growth rate in the presence of CO_2 as a carbon source was less than 50% of the growth rate with HCO_3^-. This decrease in growth rate is probably the result of a decline in the pH of the growth medium with CO_2 as the carbon source, despite the presence of TES buffer in the medium, since cultures supplemented with bicarbonate in TES buffer grew well at pH 7.15. Strain SF1 was also capable of photoheterotrophic growth in the presence of DCMU, utilizing glucose as a carbon source. However, growth in the dark was undetectable with glucose as both carbon and energy source. This response to dark conditions is similar to that of other unicellular cyanobacteria which cannot grow heterotrophically (15).

Of the nitrogen compounds tested, only ammonia or nitrate was used by the organism, and nitrogenase activity was not detected in medium containing either NH_4^+ or NO_3^-. Several amino acids inhibited growth as well as nitrogenase activity, suggesting that these amino acids were transported but not assimilated by the cells (Table 2).

In strain SF1, nitrogenase activity was not detectable in the dark, which is in contrast to other unicellular cyanobacterial systems reported (10, 12–14). The requirement of bicarbonate for enhancing the activity of nitrogenase in *Synechococcus* sp. strain SF1 also suggests that the endogenous carbon reserves, under our growth conditions, are not a major contributing factor for nitrogen fixation in this organism. The need for CO_2 fixation, as evidenced by the reduced rate of nitrogenase activity in the presence of HCO_3^- and DCMU, indicates that photosystem II is not directly supplying reductant for nitrogenase activity (Table 4). This notion of fixed carbon utilization for dinitrogen fixation is further strengthened by the observation that the nitrogenase activity detected in the presence of glucose, fructose, and sucrose was not influenced by DCMU addition. It is interesting that although fructose supported

nitrogenase activity in the presence of DCMU (Table 4), it failed to stimulate growth (Table 1). The observed enhancement of nitrogenase activity by either pyruvate or glucose is in agreement with reports on the utilization of organic compounds to support nitrogenase activity in other nitrogen-fixing cyanobacteria, including heterocystous species capable of photoheterotrophic growth (15, 20, 24). Cyclic photophosphorylation obviously supplies the ATP needed, an interpretation supported by the high nitrogenase activity of the samples containing DCMU.

The cells used in these experiments were grown in the presence of ammonium ions. After an induction period of 12 to 24 h, similar to other reports (11), it yielded a specific activity for nitrogenase which was about 1/2 to 1/4 of the respective specific activity of *Anabaena variabilis* in heterocysts only. To obtain this high specific activity, only the addition of bicarbonate was required, under conditions of optimum light intensity (Fig. 3).

All nitrogen-fixing bacteria and cyanobacteria investigated to date have been found to contain both nitrogenase and hydrogenase activities. The presumptive physiological function of the nitrogenase-linked hydrogenase is to recoup the H_2 generated during N_2 reduction by the activity of a hydrogen uptake hydrogenase (7). In *Synechococcus* sp. strain SF1, nitrogenase and hydrogenase activities were induced simultaneously after depletion of ammonia from the medium and maintained a constant activity ratio (Fig. 1). The production of HUP hydrogenase by the cell apparently required the same conditions as needed for nitrogenase.

If the role of HUP activity is to protect the nitrogenase by consuming oxygen, a lower specific activity of HUP would consequently result also in reduced activity of nitrogenase due to inactivation by oxygen. This indeed was observed in these experiments (Fig. 3). The appearance of this HUP hydrogenase activity is different from the hydrogenase activity found in vegetative cells of *A. variabilis*, which exhibited a "reversible" hydrogenase activity which was sensitive to both oxygen and light (19). The presence of HUP activity in air-grown cultures devoid of fixed nitrogen is a physiological similarity that strain SF1 shares with heterocystous cyanobacteria. Therefore, further studies with this organism, in which all the components for photosynthetic oxygen evolution as well as dinitrogen reduction are located within a single cell, may provide valuable insights into the physiology of N_2 fixation by cyanobacteria.

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