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A MUTANT OF ANABAENA SP. CA WITH OXYGEN-SENSITIVE NITROGENASE ACTIVITY[1]

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SUMMARY: Studies on the O_2 protection mechanism for nitrogenase in a mutant (PM10) of Anabaena sp. CA indicated that the ability to protect nitrogenase from O_2 was functionally impaired. Growth rates of PM10 were substantially improved when cells were cultured under microaerobic conditions. Nitrogenase activity was totally inhibited by exposure to O_2 for 30 min; partial restoration of activity was attained when cell suspensions were subsequently made microaerobic. Experiments in which induction of nitrogenase activity was followed indicated that the synthesis of the O_2 protection mechanism was temporally separated from synthesis of heterocysts and nitrogenase.

INTRODUCTION

Filamentous cyanobacteria (blue-green algae) have the capacity to fix molecular nitrogen aerobically in specialized cells known as heterocysts (1-4). However, the exact mechanism by which these specialized cells protect the O_2-labile nitrogenase from inactivation by O_2 is at present unknown (5). Recently, mutants of Anabaena sp. CA were obtained that grew poorly under aerobic conditions in a nitrogen-free medium (J.W. Gotto, unpublished observations). Preliminary results obtained in this laboratory with one of these mutant strains indicated that growth in a nitrogen-free medium could be enhanced when the organism was cultured under microaerobic conditions in an atmosphere of 98% N_2/2% CO_2. These results suggested that such mutants may be altered in their ability

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to protect nitrogenase from O_2. In this investigation, we examined the relationship of oxygen and nitrogenase in one of these mutants, strain PM 10.

MATERIALS AND METHODS

Anabaena sp. CA and a NTG[4]-derived mutant, strain PM 10, were routinely cultured in ASP-2 medium as previously described (6). Microaerobic growth was conducted in growth tubes fitted with white rubber stoppers, through which two glass tubes were inserted; one tube extended to the bottom of the growth vessel. Rubber tubing was fitted onto the glass tubes extending from the top of each rubber stopper such that the atmosphere in the vessel could be sealed off during the time tubes were not being gassed; this prevented any exchange of the atmosphere in the culture tube with that in the laboratory. Microaerobically grown cultures were continuously bubbled with a stream of N_2/CO_2 (98:2; V/V) through the long tube with the shorter tube serving as an exhaust vent. Samples from microaerobically grown cultures were taken with a gas-tight syringe to which was fitted a piece of small-bore tubing bearing an 18 guage needle, a unit long enough to extend to the bottom of the culture tubes.

Nitrogenase activity was measured by the acetylene reduction technique (7). Cell samples (2 ml) were injected into stoppered 22 ml capacity serum vials (Kimble No. 15105-L) containing an atmosphere of either argon, acetylene and CO_2 (86.5:12.5:1; V/V) or argon, O_2, acetylene and CO_2 (64.0:22.5:12.5:1; V/V). Details of incubation, sampling and detection have been described previously (8).

Samples for protein estimation were handled as follows: Aliquots (5-10 ml) of each culture were harvested by centrifugation (4°C) at 11,500 x g for 10 minutes. The pellets were washed once with 5 ml distilled-deionized water and then dried overnight under a gentle air stream. The pellets were then suspended in 2.5 ml 0.1 N NaOH containing 2% Na_2CO_3 (W/V) and the protein solubilized at 100°C for 15-20 minutes. Appropriate subsamples were taken and protein determined by the method of Lowry et al. (9). Crystalline bovine serum albumin was used as a standard.

An O_2-scavenging system consisting of glucose, glucose oxidase and catalase was used to create anaerobic conditions in some cell suspensions of PM 10 during nitrogenase assays (see Table I). The reaction was started by the addition of glucose oxidase to the vials after all other components had been added. All components of the O_2-scavenging system were made up in ASP-2 medium devoid of added combined nitrogen, except catalase, which was prepared in 50 mM K_2HPO_4, pH 7.5. By means of oxygen electrode measurements, the efficiency of O_2 removal by the scavenging system was shown to be greater than 98 percent within 3 min for a fully aerobic suspension of PM 10 cells. This level of O_2 could be maintained for at least 1 hr.

RESULTS

A comparison of N_2-dependent growth of wild-type Anabaena sp. CA and mutant PM 10 under aerobic and microaerobic conditions is shown in Fig. 1. A generation time of 4.8 hours was observed with CA when grown under either aer-

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[4] Abbreviation used is: NTG, N-methyl-N'-nitro-N-nitrosoguanidine.

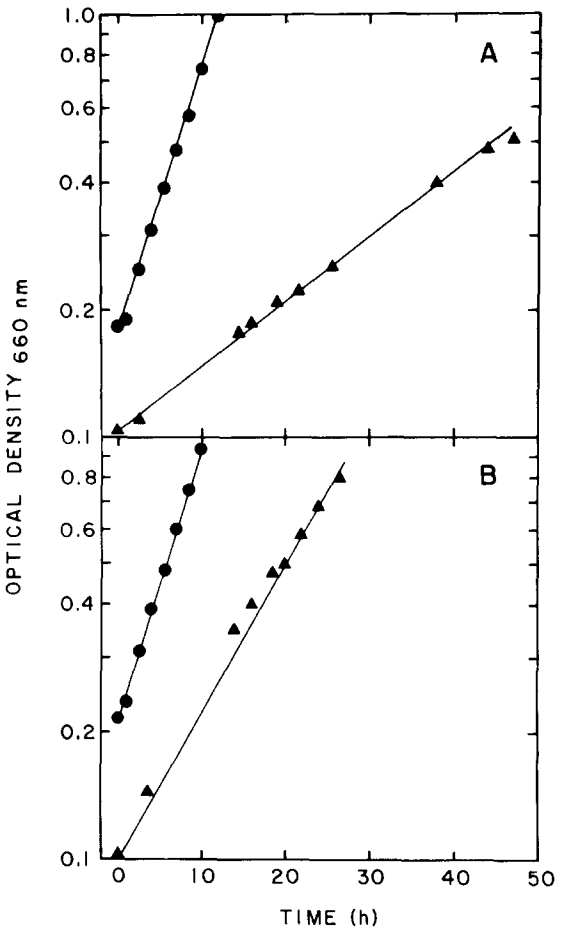

Fig. 1. Growth of Anabaena sp. CA (●) and mutant PM 10 (▲) in ASP-2 medium without combined nitrogen under aerobic (A) and microaerobic (B) conditions. CA cells were grown at 39°C on N_2 to late exponential phase through two transfers then used to inoculate ASP-2 medium without combined nitrogen pre-equilibrated with either the aerobic or microaerbic atmospheres for 1 hour prior to inoculation. PM 10 cells were grown at 39°C to late exponential phase in ASP-2 medium containing 10 mM $NaNO_3$, harvested, washed once in sterile medium minus combined nitrogen then used to inoculate pre-equilibrated nitrogen-free ASP-2 medium. Cells were subcultured once in medium of the same composition then the growth rates determined. Growth conditions were described in Materials and Methods.

obic or microaerobic conditions. In contrast, microaerobic growth of PM 10 was significantly faster than growth under fully aerobic conditions. Generation times of 9.0 hours were observed when PM 10 was cultured in CO_2-enriched

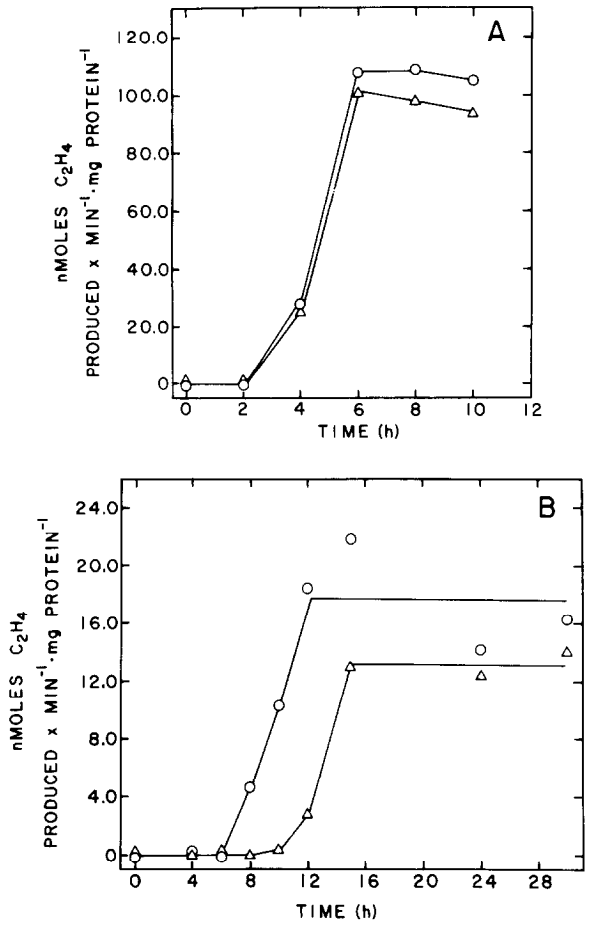

Fig. 2. Induction of nitrogenase activity in Anabaena sp. CA (A) and mutant PM 10 (B). Cells were grown at 39°C in ASP-2 medium containing 10 mM $NaNO_3$, harvested, washed once in sterile medium without added combined nitrogen and used to inoculate two 50 ml portions of preconditioned nitrogen-free ASP-2 medium. Initial protein content for suspensions of CA was 0.021 mg/ml and 0.045 mg/ml for PM 10. Samples were removed at various times for nitrogenase assays and protein determinations as described in Materials and Methods. (o), argon, acetylene and CO_2; (Δ), argon, O_2, acetylene and CO_2.

N_2 whereas generation times of approximately 20.0 hours were observed for aerobically grown cultures.

The growth data suggested that PM 10 is somehow impaired in its capacity for aerobic N_2-dependent growth. Since PM 10 grew normally on combined nitrogen ($NaNO_3$ and NH_4Cl), further experiments were designed to learn more about

the apparent inability of PM 10 to protect its nitrogenase from O_2. The kinetics of nitrogenase induction and O_2 protection of nitrogenase were thus followed in CA and PM 10 cells previously grown on $NaNO_3$. Cells were transferred, after washing in N-free medium, to medium devoid of combined nitrogen. Both heterocyst development and nitrogenase synthesis are completely repressed by growth of these organisms on nitrate. High levels of nitrogenase activity (acetylene reduction capacity) were observed approximately 4 hours after transfer of CA filaments to medium containing no added combined nitrogen; maximum nitrogenase activity occurred about 6 hours after shiftdown (Fig. 2A). In addition, mature heterocysts were observed approximately 4 hours after transfer of cells to N-free medium. Furthermore, no significant difference in the rate of acetylene reduction was detected when cell suspensions were assayed under either argon/CO_2 or argon/O_2/CO_2. Thus, after heterocysts develop, both the nitrogenase and its O_2 protective mechanism are synthesized coordinately in wild-type cells placed in a situation where they must fix N_2 in order to grow. By contrast, the kinetics of induction in PM 10 cells are substantially different than that observed with CA. For instance, nitrogenase was not detected until 8 hours after transfer of cells to N-free medium and then only when cell suspensions were assayed under reduced O_2 tension, i.e. under an atmosphere of argon/CO_2 (Fig. 2B). Also, acetylene reducing activity was not detected when assayed in the presence of atmospheric levels of O_2 until 10 hours after shiftdown; the level detected under these conditions was only about 4% of that observed in cell suspensions assayed under argon/CO_2. Moreover, the ability to detect nitrogenase in the presence of 22.5% O_2 increased with time but the level detected was only about 60-70% of that detected under reduced O_2 tensions. These results indicate that the mechanism that protects nitrogenase from inhibition by O_2 is not coordinately synthesized with the nitrogenase in PM 10 and that the O_2 protection mechanism is functionally impaired. However, O_2 protection of nitrogenase is eventually seen in PM 10 after the maximum level of nitrogenase is reached.

Table I. Rates of acetylene reduction in mutant PM 10 under either argon/CO_2 or argon/O_2/CO_2 and the effects of an O_2-scavenging system.[a]

	Acetylene Reduction	
	nmol C_2H_4 produced · min^{-1} · mg $protein^{-1}$	
	(time after shiftdown)	
Assay Conditions[b]	10 h[c]	30 h[d]
Argon/CO_2		
Exp. 1	8.1	20.9
Exp. 2	11.4	19.1
Exp. 3	7.4	N.D.[e]
Exp. 4	9.3	21.1
Argon/O_2/CO_2		
Exp. 1	0.1	15.9
Exp. 2	0.1	8.4
Exp. 3	0.0	N.D.[e]
Exp. 4	4.4[f]	12.3[f]

[a]Growth and subsequent handling of cells were as in Fig. 1.

[b]Exp. 1 contained cells suspended in ASP-2 medium; Exp. 2 contained cell suspension and 0.21 mM glucose; Exp. 3 contained cells and 7.5 units of glucose oxidase and 6,500 units catalase; Exp. 4 contained cells, 0.21 mM glucose and 7.5 units of glucose oxidase and 6,500 units of catalase.

[c]Each vial received a total of 0.155 mg protein.

[d]Each vial received a total of 0.240 mg protein.

[e]Not determined.

[f]Rates calculated on data collected during initial 45 minutes of reaction.

Although the kinetics of induction of heterocysts, nitrogenase activity, and O_2 protection of nitrogenase are different in CA and PM 10, nitrogenase activity is not detected in either organism until mature heterocysts are observed microscopically. In addition, both strains develop heterocysts to a frequency of approximately 10%.

The above results indicate that the expression of nitrogenase activity in PM 10 is inhibited but not irreversibly damaged by O_2, especially during the time immediately following heterocyst development.

Since samples taken from aerobic cultures and subsequently placed in vials containing argon/CO_2 still contain substantial dissolved O_2, experiments were conducted with cell suspensions made anaerobic using an O_2-scavenging system. Results with a glucose oxidase-catalase system are shown in Table I. As observed previously, nitrogenase activity could be detected 10 hours after shiftdown in suspensions assayed under greatly reduced O_2 tensions (argon/CO_2) but not in suspensions assayed under argon/O_2/CO_2. Creation of anaerobic conditions by the O_2-scavenging system in suspensions assayed under argon/CO_2 did not lead to higher nitrogenase activities. However, institution of anaerobiosis in a suspension initially under an atmosphere of argon/O_2/CO_2 resulted in restoration of approximately 48% of the activity observed with suspensions under argon/CO_2. As was observed in previous experiments, O_2 protection of nitrogenase was apparent 30 hours after shiftdown and the rate of acetylene reduction observed in suspensions where the O_2 tension was reduced was about 1.3 times higher than in suspensions assayed under argon/O_2/CO_2.

Since microaerobic growth of PM 10 cells is substantially faster than that observed for cells grown under aerobic conditions, the effects of oxygen on nitrogenase activity in cells grown in CO_2-enriched N_2 were determined (Table II). The results of this experiment show that atmospheric levels of O_2 have an immediate inhibitory effect on the nitrogenase in microaerobically grown CA. However, all of the activity is regained when microaerobic conditions are re-established. Since the rate of acetylene reduction was linear for the entire time after the cell suspension was made microaerobic, new protein synthesis is probably not required to restore full microaerobic nitrogenase activity. In contrast, treatment of microaerobically grown PM 10 cells with atmospheric levels of O_2 caused an immediate and complete inhibition of nitrogenase activity. However, approximately 40% of the original activity was restored when the cell suspension was made microaerobic.

DISCUSSION

In this study the effects of free oxygen on nitrogen fixation and growth of Anabaena sp. CA and mutant strain PM 10 were investigated. Our results in-

Table II. Reversibility of O_2-inhibition of nitrogenase in Anabaena sp. CA and mutant PM 10 grown under microaerobic conditions.

Assay Conditions[b]	Acetylene Reduction[a] nmole C_2H_4 produced · min^{-1} · mg protein^{-1}	
	Anabaena sp. CA[c]	PM 10[c]
1. Argon/CO_2	137.0	73.1
2. Argon/O_2/CO_2	91.4	0.9
3. Argon/CO_2 ↓ Argon/O_2/CO_2 ↓ Argon/CO_2	110.3 54.8 133.6	59.1 0.0 23.3

[a] CA and PM 10 were grown through 2-3 transfers in nitrogen-free ASP-2 medium. Acetylene reduction rates were established over a period of 75 min and were linear in all cases.

[b] Rates of acetylene reduction for each organism were established for 40 min in an atmosphere of argon/CO_2 after injection of cells into assay vials. At 42 min, O_2 was injected into each vial to a final concentration of 22.5%. After equilibration for 3 min, excess pressure was relieved and acetylene reduction followed for 30 min. At the end of this time, illumination was discontinued and the cell suspension in each vial made anaerobic by gentle bubbling of a stream of argon/CO_2 for 8 min. Acetylene was then added to each vial to a concentration of 12.5%; after 3 min equilibration, excess pressure was relieved, the cell suspension illuminated and acetylene reduction followed for an additional 60 min.

[c] 0.112 mg CA cell protein and 0.129 mg PM 10 cell protein were used.

dicate that the O_2 protective mechanism for nitrogenase in PM 10 is functionally impaired for several reasons. First, culturing cells under microaerobic conditions resulted in growth rates that were substantially faster than that observed in aerobically grown cells. Second, experiments where the induction of nitrogenase in aerobic cultures was followed indicated that synthesis of the protective mechanism is temporally separated from the synthesis of heterocysts and nitrogenase. Also, a comparison of the growth and acetylene reduction data showed that growth of cells and protein accumulation under aerobic conditions did not resume until maximum rates of acetylene reduction as assayed in vials containing argon/O_2/CO_2 were detected. Third, in experiments not reported here, aerobic growth of PM 10 on combined nitrogen is normal, which rules out the possibility that nitrate and ammonia assimilatory mechanisms are damaged in this

organism. Finally, although nitrogenase activity in microaerobically grown PM 10 cells was immediately and totally inhibited by short exposure to atmospheric levels of O_2, restoration of activity to a level normally observed in aerobically grown cultures was attained upon return to microaerobic conditions.

These results thus indicate that nitrogenase in PM 10 is afforded only limited protection from the deleterious effects of O_2. However, our results can not unequivocally distinguish between an impaired ability to protect nitrogenase from O_2 damage or an inability of some system to properly transfer electrons to the nitrogenase complex. Experiments are at present underway to attempt to resolve these two possibilities. Strain PM 10 is of particular interest for these studies since the capacity for aerobic nitrogen fixation is gradually regained in this strain. Thus, the events surrounding the development of the O_2 protection mechanism might be studied.

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