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STUDIES ON THE REGULATION OF NITROGEN FixATION IN CYANOBACTERIA, WITH SPECIAL REFERENCE TO THE EFFECTS OF OXYGEN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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*****

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1996

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ABSTRACT

The aim of this dissertation was to gain a further understanding of the intracellular components and strategies that two diverse strains of nitrogen-fixing cyanobacter employ to cope with oxygen. The unicellular strain *Synechococcus* sp. strain RF-1 exhibited a circadian rhythm of N₂ fixation when grown under diurnal light-dark cycles. The heterocystous strain, *Anabaena* sp. strain CA, did not exhibit such a rhythm but its nitrogenase recovered from oxygen-mediated inactivation even when whole filaments were exposed to an atmosphere of 1% CO₂/99% O₂. In this study, the pattern of *nifH* and *rbcL* transcription and the pattern of Fe protein modification in *Synechococcus* RF-1 were examined during diurnal light-dark cycles and also under continuous light conditions. When grown under continuous light conditions, *nifH* and *rbcL* were continuously transcribed. When grown under 12 h light/12 h dark conditions, *nifH* transcripts were present mainly in the dark, while *rbcL* was transcribed both in the dark and in the light, but with significantly greater expression during the light cycles. The appearance of the Fe protein during the dark cycles correlated with nitrogenase activity. To probe the mechanism by which *Anabaena* CA protects/reCOVERS its nitrogenase from O₂-mediated inactivation, several approaches were applied to identify potential proteins and genes that might be involved in the protection/recovery of O₂-mediated inactivation of nitrogenase in *Anabaena* CA. Nine O₂-sensitive mutants of CA that might lack the protection/recovery system were isolated by NTG mutagenesis. Comparisons of the protein profiles of wild-type CA and O₂-sensitive mutant N102 obtained from two-dimensional gel analysis indicated there were at least 15 proteins that might be involved in the recovery/protection process. In this study, subtractive hybridization was also used to identify genes of CA that were differentially expressed under hyperbaric O₂ conditions.
To my parents
ACKNOWLEDGEMENTS

I express sincere appreciation to my adviser Dr. F. Robert Tabita for his guidance, support and help throughout the research. Thanks go to the other members of my advisory Committee, Drs. Charles J. Daniels, Joseph A. Krzycki, and William R. Strohl for their time, comments and suggestions. Thanks also go to Drs. Janet L. Gibson, Ted Lee, Greg Watson, Howard Xu, Jim Dubbs, and George Paoli, for their suggestions, discussion and technical help. Finally, to my mother, brothers and sisters, I express my thanks for your support and encouragement.
VITA

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CHAPTER I
INTRODUCTION

The reduction of N₂ to ammonia is catalyzed by nitrogenase, an enzyme complex composed of the Mo-Fe and Fe proteins. The Mo-Fe protein of nitrogenase (dinitrogenase) is a tetramer (α₂β₂) composed of two different subunits with a molecular weight of about 220,000 Da containing four (4 Fe-4S) clusters and two molecules of the Fe-Mo cofactor (FeMoCo). The Fe protein, dinitrogenase reductase, is a dimer (γ₂) of two identical subunits with a single (4Fe-4S) cluster and a molecular weight between 57 kD and 72 kD depending on the source of the protein (16). The nifH, nifD, and nifK are the structural genes of nitrogenase; nifH codes for dinitrogenase reductase (γ subunit), while nifD and nifK code for the α- and β- subunits of dinitrogenase, respectively. Regardless of the source of nitrogenase, the enzyme complex from various diazotrophs is found to be highly conserved. It is believed that FeMoCo in dinitrogenase is the actual site where N₂ binding and reduction take place. The reduction of N₂ to ammonia catalyzed by nitrogenase is a highly endergonic reaction; the reduction of 1 mol of N₂ to 2 mol of NH₃ requires about 12 to 16 mol of ATP (16,28). The reducing equivalents for this reduction are supplied by dinitrogenase reductase. In most diazotrophs, ferredoxin or flavodoxin (under conditions of iron limitation) are thought to be the immediate electron donors to nitrogenase. The ultimate source of electrons for N₂ fixation is derived from cell metabolism (10).

One unique property of the nitrogenase complex is its extreme O₂ sensitivity, regardless of the source. Exposure to O₂ damages both dinitrogenase and dinitrogenase reductase (16,80,103), with dinitrogenase reductase the more sensitive of the two proteins, having a half-life in air around 30 s to 2 min. The half-life of dinitrogenase is between...
4.5 min to 10 min (25,111). It is thought that the damaging effects of O\textsubscript{2} to the nitrogenase complex might be due to reactive O\textsubscript{2} species generated during the reduction of O\textsubscript{2} (28,31,48,61,103). These reactive O\textsubscript{2} species could either change the structure or the redox state of this enzyme complex and the 4Fe-4S clusters, hence, making the protein incapable of accepting or donating electrons (103,114). O\textsubscript{2} might also destroy the nitrogenase complex by initiating protease activity, which may in turn causing the rapid degradation of nitrogenase (28).

Cyanobacteria, also called blue-green algae, are a unique group of gram-negative phototrophs. They have existed on earth for more than 2.5 billion years according to fossil records. Based on cell morphology and cell division, cyanobacteria can be categorized into five groups: unicellular binary fission, unicellular multiple fission, filamentous nonheterocystous, filamentous heterocystous, and branched filamentous heterocystous strains (100). Photosynthesis in cyanobacteria is found to be similar to that of eukaryotic algae and plants in that both contain photosystems I and II and are capable of photolyzing water to generate O\textsubscript{2} as a photosynthetic byproduct. The capacity for aerobic N\textsubscript{2} fixation has been demonstrated in a variety of cyanobacteria including unicellular, filamentous nonheterocystous, and heterocystous strains. To prevent nitrogenase from O\textsubscript{2} damage, these organisms must develop some strategy to protect the enzyme from O\textsubscript{2} generated from photosynthesis as well as from the atmosphere. Several mechanisms have been hypothesized in non-heterocystous and heterocystous strains to explain how damage of O\textsubscript{2} to the nitrogenase complex, may be limited, such as the rapid replacement of damaged nitrogenase by new synthesis of enzyme (9), respiratory removal of O\textsubscript{2} (27,45,73), temporal separation of N\textsubscript{2} fixation and photosynthesis in nonheterocystous and unicellular strains (34,35,78,79), spatial separation of N\textsubscript{2} fixation and photosynthesis in heterocystous strains (136), and conformational protection (posttranslational modification of dinitrogenase) in Anabaena spp. (115). The spatial differentiation in heterocystous
strains allows these organisms to perform two incompatible reactions in two types of cells under aerobic conditions; N2 fixation is carried out in differentiated cells called heterocysts, while oxygenic photosynthesis takes place in the vegetative cells. The heterocyst is the sole site for N2 fixation under aerobic conditions, although it has been reported that in strains where the development of mature heterocysts is arrested; i.e. in some *Nostoc* and *Anabaena* species, N2 may be fixed under anaerobic conditions (100). It is thought that the unique structure of heterocysts provides some diffusion barrier to O2 and other gases, therefore allowing N2 fixation to take place in heterocysts under aerobic conditions. Heterocysts have a thick envelope which consists of three layers; an inner laminated layer; a homogenous central layer; and a fibrous outer layer. The laminated layer consists of a mixture of glycolipids that is unique to the heterocyst envelope. Mutants of *Anabaena variabilis* deficient in heterocyst glycolipids showed an oxygen sensitive phenotypes and could only fix N2 under microaerobic conditions, supporting the idea that these heterocyst-specific glycolipids contribute to a diffusion barrier for O2 (49,63). The central homogenous layer and outer fibrous layer of the heterocyst envelope consists of polysaccharide. These polysaccharide layers help to maintain the integrity of the glycolipid layer or may also have some function in impeding the diffusion of atmospheric O2 into heterocysts.

A respiratory protection mechanism has also been hypothesized to help stabilize nitrogenase from inactivation by O2. This idea was suggested because there is a higher amount of cytochrome aa3 oxidase in heterocysts compared to vegetative cells (51). Also, there appears to be a correlation between the glycogen content of filaments and the stabilization of nitrogenase to O2 inactivation (25,45). Another proposed mechanism for O2 protection in heterocystous strains is that a hydrogenase mediated oxyhydrogen (Knallgas) reaction occurs within the heterocysts. However studies with wild-type and
mutant strains of *Anabaena* strain CA indicated this was not sufficient to protect nitrogenase from O\(_2\) (28,117).

Finally, it was hypothesized that a mechanism similar to conformational protection of nitrogenase in *Azotobacter* spp. (103) may be operable in *Anabaena* sp. CA (93,115,125). *Anabaena* sp. strain CA is a unique marine heterocystous strain that exhibits a rapid growth rate (118) and synthesizes heterocysts and nitrogenase in the presence of ammonia (11). Previous experiments of *Anabaena* sp. strain CA demonstrated that the initial inactivation of nitrogenase activity under an atmosphere of 1% CO\(_2\)/99% O\(_2\) was followed by the recovery of nitrogenase activity. It was also found that hyperbaric O\(_2\) treatment results in a decrease in the mobility of dinitrogenase reductase subunits. The molecular weight of this protein on SDS gels was changed from 36 kD to 38 kD. This alteration is thought to be due to a reversible posttranslational modification (115).

Posttranslational regulation of nitrogenase activity by modifying the Fe-protein of nitrogenase has been reported in a variety of cyanobacteria. It has been suggested that nitrogenase regulation in cyanobacteria might follow the Fe-protein ADP-ribosylation pattern described for *Rhodobacter capsulatus* and *Rhodospirillum rubrum*. In these organisms, a single subunit of the Fe-protein is ADP-ribosylated. However, this has been shown not to be the case in cyanobacteria. Firstly, inactivation of the Fe-protein in various cyanobacteria, including *Anabaena variabilis*, *Anabaena* sp. strain CA and *Synechococcus* sp. strain RF-1 has been shown to result from the modification of both subunits of the Fe protein. Secondly, the modified Fe protein of *R. rubrum* cross-reacts with antibody that is specific for ADP-ribose, but the enzymes from *Anabaena variabilis* and *Synechocystis* BO8402 do not (137). These results suggest that modification of dinitrogenase reductase in cyanobacteria is not due to ADP-ribosylation. It is possible that the observed alteration of the Fe-protein is a non-enzymatic process. The transitions seen on gels might be simply due to the consequence of a change in the overall net charge of the Fe-protein, since Smith
et al (115,125) found that metranidizole-treated cell suspension of *Anabaena* CA (presumably under highly oxidizing conditions) also showed the alteration of Fe-protein on gels. The mechanism for the modification of the Fe protein in cyanobacteria is not clear. However, in *Anabaena* CA, this modification correlated with the tolerance of nitrogenase to O₂ (93,115,125). The recovery of nitrogenase activity by strain CA required new protein synthesis but was not completely dependent on de novo synthesis of nitrogenase (93). The metabolism of ammonia, but not nitrate, was also found to repress the recovery process (116); this is opposite to the effect of these fixed nitrogen compounds on nitrogenase synthesis (93,116).

The purpose of this dissertation was to gain a closer understanding of the mechanism by which cyanobacteria protect their nitrogenase from the deleterious effects of O₂ by using two diverse strains, a unicellular cyanobacterium, *Synechococcus* sp. RF-1, and a heterocystous strain, *Anabaena* CA. In studies with *Synechococcus* sp. strain RF-1 grown under diurnal light/dark cycles, the transcriptional and translational products of *nifH* were found mainly during the dark phase, while the transcription of *rbcL*, encoding Rubisco, one of the key enzymes of CO₂ assimilation, exhibited greater expression in the light than in the dark. These results provide experimental evidence for the temporal separation of N₂ fixation and photosynthesis in *Synechococcus* RF-1. For cultures grown under light/dark conditions, this study also indicated that the nitrogenase activity present during the dark phase resulted from de novo synthesis of nitrogenase. During the course of probing the mechanisms by which *Anabaena* sp. strain CA recovers/protects its nitrogenase activity from O₂-mediated inactivation, several intriguing results were obtained. Nine oxygen sensitive mutants of *Anabaena* CA were isolated. They were unable to grow aerobically or grew poorly under nitrogen-fixing conditions. By comparing the 2D gel electrophoretic protein profiles of wild-type strain CA and an oxygen sensitive mutant N102, fifteen proteins were identified. Using a subtractive hybridization strategy,
several up-regulated genes under hyperbaric O$_2$ conditions have been identified which might be involved in encoding products which contribute to thickening of the cell envelope of heterocysts. In addition, genes involved in N$_2$ fixation itself, and genes important for electron transport, O$_2$ scavenging or ATP production were also identified which may contribute to aerobic N$_2$ fixation.
CHAPTER II
Reciprocal Light/Dark Transcriptional Control of nif and rbc Expression and Light-Dependent Post translational Control of Nitrogenase Activity in Synechococcus sp. Strain RF-1¹

INTRODUCTION

Since cyanobacteria undergo oxygenic photosynthesis, it is not surprising that these organisms have evolved diverse mechanisms for protecting nitrogenase from inactivation by O₂. In heterocystous cyanobacteria, protection is achieved by confining nitrogenase to the heterocysts (28,47,136), differentiated cells that somehow offer an environment free from the deleterious effects of oxygen (29). For non-heterocystous filamentous and unicellular nitrogen-fixing cyanobacteria, different mechanisms have been proposed to explain how nitrogenase is protected from inactivation by O₂, including temporal separation of N₂ fixation and photosynthesis, O₂ consumption, and respiration (29,36,44,79,95,119,121).

In various photosynthetic bacteria (41,56,94), non-photosynthetic Azospirillum spp. (32), and several cyanobacteria (12,90,91,95,115,120), nitrogenase activity is controlled via posttranslational regulation and modification of the Fe protein of nitrogenase. In the nonsulfur purple photosynthetic bacteria Rhodospirillum rubrum and Rhodobacter capsulatus, reversible posttranslational modification is catalyzed by two enzymes which act to reversibly modify and de-modify one of the two subunits of the Fe protein of

¹This chapter has been published in J. Bacteriol.
nitrogenase (56,68,69,106,107). In cyanobacteria, modification of the Fe protein was first reported in *Anabaena* sp. strain CA as a response to O2. Since these results were first reported, alteration of the Fe protein has also been reported in several other heterocystous and non-heterocystous cyanobacteria under a variety of conditions (12,90,91,96,120), however the nature of the modification and the mechanism by which it occurs remains elusive.

In order to gain an understanding of the mechanism by which nitrogenase is regulated in cyanobacteria, we have initiated studies with *Synechococcus* sp. RF-1, an organism with a well-described pattern of nitrogen fixation (43,44,52-54). This unicellular N2-fixing cyanobacterium exhibits a Circadian rhythm of N2-fixation such that both nitrogenase activity and *nif* gene transcription occurs only in the dark during a light-dark diurnal growth regimen (43,52-54). In this study, the products of *nifH* and *rbcL* transcription and *nifH* translation were examined during 12 h light/12 h dark and continuous light conditions. Evidence for Fe protein modification and its subsequent degradation in the light phase was clearly evident, indicating that the nitrogenase activity found in the dark phase is due completely to de novo synthesis of nitrogenase polypeptides. The transcription of *rbcL*, which encodes the large subunit of RubisCO, the key enzyme of light-dependent CO2 fixation, also appears to be regulated during light/dark shifts, however in a reciprocal manner.

**MATERIALS AND METHODS**

**Growth conditions.** Axenic cultures of *Synechococcus* sp. strain RF-1 (PCC 8801) were grown in nitrogen-free BG-11 medium, supplemented with 10 mM EPPS [N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid] buffer (pH 8.0). The cultures were incubated in either continuous light, or a 12 h light/12 h dark cycle, as described
previously (52). In all instances, the organism was cultured for at least 14 complete light/dark cycles before samples were analyzed.

**Assay of nitrogenase.** The acetylene reduction assay was used to measure nitrogenase activity (23). Samples of 2 ml (at a cell density of about $1 \times 10^7$ cells/ml) were withdrawn from cultures at 2 h intervals over a 24 h period from a culture grown in 12 h light-12 h dark (1000 to 2200 h) cycle. The samples were added to 24 ml vials sealed with serum stoppers, followed by the injection of 2.4 ml of acetylene. Gas samples (1 ml) were analyzed for their ethylene content at the beginning of the incubation period and 1 h later using a Varian 3300 gas chromatograph (Varian Associates, Inc., Sunnyvale, California) equipped with a flame ionization detector and Poropak N column. Prior control experiments had established that ethylene production was first order with respect to time and that a 1 h reaction time was suitable for assays.

**Western (immunoblot) identification of Fe protein.** Cultures of *Synechococcus* sp. RF-1 (30 ml), grown under a 12 h light - 12 h dark (1000 to 2200 h dark) regimen, were withdrawn at 2 h intervals over 24 h. Cells were harvested by centrifugation and resuspended in 700 μl of sonication buffer (50 mM Tris-HCl, pH 6.8, 1 mM EDTA, 14 mM 2-mercaptoethanol). Cells were broken with a Heat Systems W-385 sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY). Cell extracts, containing about 60 μg protein, were suspended in sodium dodecyl sulfate (SDS)-sample buffer, boiled for 2 min, and loaded onto a SDS-polyacrylamide (SDS-PAGE) gel containing 12.5% acrylamide (70). Fractionated proteins were then electroblotted onto a nitrocellulose filter (Millipore Corp., Bedford, MA) in 25 mM Tris-Cl (pH 8.3)-192 mM glycine-20% methanol with a Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Richmond, CA) (130). Antiserum against the Fe protein of nitrogenase from *Rhodospirillum rubrum* (kindly provided by P.W. Ludden), was diluted 1:200 and used to identify the Fe protein of nitrogenase, as previously described (115).
Extraction of RNA and Northern blot analysis. Samples (30 ml) of Synechococcus sp. RF-1, grown under both 12 h light/12 h dark (800 to 2000 h light) or continuous light regimes, were withdrawn at 2 h intervals over a 24 h time period. RNA was extracted and analyzed as described previously (52,53). Each sample, containing 10 mg RNA, was fractionated on formaldehyde gels. Nitrogenase-specific mRNA and RubisCO-specific mRNA were detected by hybridization with \(^{32}\text{P}\)-labeled \textit{nifH} (using plasmid pAn154.3) (98) and \(^{32}\text{P}\)-labeled \textit{rbcL} (from plasmid pBGL520) (64) probes prepared by nick translation (99). Quantitation of \textit{nifH} and \textit{rbcL} gene transcription on Northern blots was determined using a Betagen analyzer (Betascope 603 Blot analyzer, Mountain View, Calif.).

RESULTS AND DISCUSSION

Transcription of \textit{nifH} and \textit{rbcL} genes in \textit{Synechococcus} sp. RF-1. Samples taken from standing cultures grown in continuous light were examined for ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) specific mRNA using a \textit{rbcL} specific probe (Fig. 1A). The same blots were stripped and then rehybridized with a \textit{nifH} probe to identify mRNA specifying the Fe protein of nitrogenase. Under continuous light conditions, \textit{nifH} transcripts of 4.5, 3.2 and a 1.3 kb were detected throughout the 24-h experimental period (Fig. 1C), a pattern consistent with previous studies (53). Similar to \textit{nifH}, the \textit{rbcL} gene of \textit{Synechococcus} RF-1 was continuously transcribed throughout the 24 h experimental period (Fig. 1A and 1C), as was the \textit{rbcS} gene (data not shown), which is cotranscribed with \textit{rbcL} in all prokaryotes thus far examined (124). For \textit{nifH} and \textit{rbcL}, maximum transcription was seen at 2100 and 2300 h (Fig. 1B, 1D). A reproducible enhancement of \textit{nifH} and \textit{rbcL} transcription was also seen at hour 1100 and 700, respectively; the reason for this increase in \textit{nifH} transcription 1100 and 2100 to 2300 and
Fig. 2.1. Northern blot analysis and nifH transcription in *Synechococcus* sp. strain RF-1 grown under continuous light. Cells from a culture incubated in a cycle of 12 h of light and 12 h of dark were inoculated into fresh growth media and cultured for at least 2 weeks under continuous-light conditions. Samples were withdrawn at 2-h intervals over 24 h from a standing continuously illuminated culture. For the detection of RubisCO-specific mRNA, 32P-labeled rbcL (from plasmid pBGL520) (64) was used as a probe (A). The blot was then stripped and reprobed with a nifH probe (from plasmid pAn154.3) (98) to identify Fe protein-specific mRNA (C). In all cases, 10 µg of RNA was loaded onto the gel. The relative amounts of *rbcL* and *nifH* gene transcription were determined with a Betagen analyzer (B and D).
Fig. 2.2. Northern blot analysis of *rbcL* and *nifH* transcripts in *Synechococcus* sp. strain RF-1 grown under a cycle of 12 h of light and 12 h of dark. Samples were withdrawn at 2-h intervals over 24 h from a standing culture which had been previously adapted to at least 14 cycles of a regimen of 12 h of light (800 to 2000 h) and 12 h of dark (2000 to 800 h). Transcripts were analyzed as described in the legend to Fig. 2.1 with *rbcL* (A) and *nifH* (C) probes. In all cases, 10 μg of RNA was loaded onto the gel. The relative amounts of *rbcL* and *nifH* gene transcription were determined with a Betagen analyzer (B and D).
the increase in \(\text{rbcL}\) transcripts at 700 and 2100 and 2300 is not understood at this time but may reflect an attempt by the cell to maintain some balance in the two reductive processes of \(\text{N}_2\) fixation and \(\text{CO}_2\) fixation during the cell cycle under continuous light.

Cultured under the conditions of alternating 12 h of light and 12 h of dark, \(\text{nifH}\) transcripts were present mainly in the dark (Fig. 2C and Fig. 2D), in agreement with the results of previous studies (52). However, in contrast to nitrogenase, \(\text{rbcL}\) transcripts (of 2.9, 2.4 and 1.6 kb) were detected in both the light and in the dark, with considerably greater expression in the light phase (Fig. 2A and Fig. 2B). These results are intriguing in light of previous studies which suggested a temporal separation of \(\text{N}_2\) fixation in the dark and photosynthesis in the light in \(\text{Synechococcus}\) spp.; indeed, this temporal separation was hypothesized to be potentially important to protect nitrogenase from the deleterious effects of photosynthetically evolved oxygen (44,79). Since RubisCO is one of the key enzymes of the reductive pentose phosphate pathway for carbon dioxide assimilation in cyanobacteria (124), the significant increase in \(\text{rbcL}\) specific mRNA in the light provides experimental verification for the temporal separation hypothesis at the molecular level. That is, similar to nitrogenase, RubisCO gene transcription is also controlled during the light-dark cycle, but in a reciprocal fashion.

**Light/dark effects on nitrogenase activity plus Fe protein modification and synthesis in \(\text{Synechococcus}\) sp. RF-1.** To determine whether posttranslational modification might contribute to the appearance and disappearance of nitrogenase activity in the dark and the light, respectively (44,52), antiserum against the \(\text{Rhodospirillum rubrum}\) Fe protein was employed to probe the electrophoretic properties of the \(\text{Synechococcus}\) sp. strain RF-1 protein during the light-dark cycle. The appearance of the Fe protein during the dark phase correlated with the pattern of nitrogenase activity (Fig. 3). In cells collected from the dark phase, two bands with molecular masses of 37 and 39 kDa, cross-reacted with the antiserum. Both bands were always found with cells which
Fig. 2.3. Nitrogenase activity (A) and immunoblot analysis (B) of the Fe protein of nitrogenase in *Synechococcus* sp. strain RF-1 grown under cycles of 12 h of light and 12 h of dark. Samples of a culture which had been previously adapted to a diurnal light-dark regimen of 12 h of light (2200 to 1000 h) and 12 h of dark (1000 to 2200 h) were withdrawn at 2-h intervals for 24 h. For each sample, nitrogenase activity in whole cells was assayed and a crude extract was subsequently prepared. Antiserum against the Fe protein of nitrogenase from *Rhodospirillum rubrum* was used to detect the Fe protein of nitrogenase.
had nitrogenase activity; however, the relative abundance of the 39-kDa protein increased at 1900 and 2100 (Fig. 3B) and appeared to correlate with the gradual loss of nitrogenase activity as cells were maintained in the dark (Fig. 3A). The rapid and total disappearance of the 37-kDa protein band when cells were illuminated for 1 h (2300 h) correlated with the complete absence of enzyme activity and the increase in the intensity of the 39-kDa protein (Fig. 3B). These results suggest that the 37-kDa protein, present exclusively in the dark, may be the active form of the Fe protein; the 39-kDa band may be the modified and inactive form of the Fe protein. Neither the 37-kDa nor the 39-kDa proteins could be detected after cells were illuminated for 3 h (100 h; Fig. 3B). The 39-kDa protein predominates in cells taken from the 2300 h; in this experiment, this is 1 h after the dark phase ends (and 1 h after the light phase begins), a time when there is no detectable nitrogenase activity (Fig. 3A). These studies indicate that nitrogenase activity present during the dark cycle results from de novo synthesis of nitrogenase; the rapid degradation of the Fe protein upon entering the light phase may result from activating or inducing a protease, which appears to be specific for the modified, and presumably inactivated, 39-kDa Fe protein. In this respect, the posttranslational modification of the Fe protein of Synechococcus RF-1 appears quite similar to the oxidative modification (or "marking") and subsequent degradation of both glutamine synthetase and RubisCO from enteric bacteria (66) and Rhodospirillum rubrum (21), respectively. By contrast to the situation with Fe protein in the light, we have not observed a reciprocal drastic loss of RubisCO activity or degradation of RubisCO protein when the cells are placed in the dark nor is there complete de novo synthesis of RubisCO protein in the light. Instead, our preliminary results suggest that preformed RubisCO is stably maintained in the dark and not degraded. Nevertheless, rbcL transcription obviously resumes in the light after a quiescent period in the dark.

In summary, these studies provide conclusive evidence for the temporal separation of rbcL and nifH transcription when Synechococcus RF-1 is incubated under light/dark
conditions. Since the appearance of these transcripts directly reflects the expression of genes that encode key enzymes of both N\textsubscript{2} fixation and CO\textsubscript{2} assimilation, it is apparent that the two reductive processes are reciprocally regulated during light-dark cycling. Presumably, this diurnal control over \textit{nif} and \textit{rbc} transcription relates to the behavior of these organisms in their native environment, which is likely to be one that involves some form of diurnal light-dark cycle. The precise molecular signals that are responsible for switching one biosynthetic system on and the other off and vice versa remain to be discovered. Moreover, there do appear to be times during the light-dark transition when significant levels of both \textit{nifH} and \textit{rbcL} transcripts are present, e.g., at 900 and 1900 to 2100 (Fig. 2). Since these times represent periods when the cell has just entered the light phase (900) or is soon to enter or just entered the dark (1900 to 2100 h) phases, perhaps the cell is adjusting its molecular regulatory circuits at these times in preparation for the upcoming transition. Indeed, since cells had been adapted to at least 14 cycles of light-dark transition before samples were removed for analysis, these data are compatible with a true circadian-like rhythm of \textit{nif} and \textit{rbc} transcription. Exactly why this organism takes the rather drastic expediency of removing all vestiges of the Fe protein after exposure to light may be related to the fact that strain RF-1 is not equipped to protect its nitrogenase from the deleterious effects of oxygen as the organism undergoes active photosynthesis. Thus it would be absolutely necessary for strain RF-1 to marshal a concerted effort to synthesize this protein \textit{de novo} when the organism is placed in the dark. In view of the importance of this commitment, perhaps it is not surprising that \textit{Synechococcus} strain RF-1 begins to accumulate \textit{nif}-specific mRNA 1 h prior to the onset of darkness.
ACKNOWLEDGMENTS

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CHAPTER III
Proteins Synthesized by Wild-type and Oxygen Sensitive Mutants of Anabaena sp. Strain CA During the Recovery of Oxygen-mediated Inactivation of Nitrogenase

INTRODUCTION

The inhibitory effect of atmospheric oxygen on nitrogen fixation was noted in early studies with free-living and symbiotic nitrogen-fixing organisms (6,46,92). Exposure to oxygen results in an irreversible inactivation of both components of the nitrogenase complex, the Mo protein (dinitrogenase) and the Fe protein (dinitrogenase reductase) (16,80,103). Although the biochemical characteristics of the highly oxygen-sensitive nitrogenase were shown to be essentially the same for all nitrogen-fixing organisms studied, the mechanisms that different organisms employ to protect the enzyme system from the damaging effects of oxygen are quite different. Since cyanobacteria perform oxygenic photosynthesis, the O₂ produced by these organisms is especially a problem to the O₂-labile nitrogenase enzyme complex. Therefore, cyanobacteria have developed diverse strategies to protect the nitrogenase complex from O₂ injury, such as differentiation of special cells, heterocysts, in some filamentous strains, and a temporal separation of photosynthesis and nitrogen fixation in unicellular cyanobacteria (18,29,36,43,47,79,95,136)

Several years ago it was found that exposure of Anabaena sp. strain CA to hyperbaric oxygen (99% - 100%) resulted in the inactivation of nitrogenase, followed by its recovery by a process that required protein synthesis. The recovery from O₂-mediated
inactivation was found not simply to be due only to de novo synthesis of nitrogenase (93). The alteration of the migration of the Fe protein of nitrogenase on SDS polyacrylamide gels from a 36 kD protein to a 38 kD protein was also found in response to O2-treatment (115). These results and other earlier studies with pleiotropic mutants of Anabaena CA (40,42) suggested a complex, dynamic process for the protection and/or recovery of nitrogenase from oxygen inactivation (116).

Two-dimensional polyacrylamide gel electrophoresis is a useful method to resolve the pattern of proteins synthesized by the cell in response to different growth conditions. This method has been successfully used to identify heat shock proteins, starvation-induced proteins, and indeed physiological manipulations in proteins synthesized in response to a variety of several organisms. Since the aim of this study was to gain a better understanding of the mechanism by which the nitrogenase of Anabaena CA is protected or recovers from O2-mediated inactivation, we investigated the changes of proteins synthesized by wild-type CA and an O2-sensitive mutant strain N102, a strain that had lost its ability to recover from O2-mediated inactivation. Several additional O2-sensitive mutants were also isolated and proved useful for these studies.

**MATERIALS AND METHODS**

**Organisms and growth conditions.** Anabaena sp. strain CA (ATCC 33047), and the oxygen-sensitive mutants of strain CA selected in this study were grown in ASP-2 medium containing 5 g/l of NaCl at 39°C with constant illumination and were bubbled with either 1% CO2 in air or 1% CO2/99% N2 as previously described (115).

**Isolation of O2 sensitive mutants of Anabaena sp. strain CA.** To isolate O2 sensitive mutants of CA, wild type strain CA was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NTG), as previously described (14,38). Mid-log phase
cultures of strain CA (20 ml) were harvested and resuspended in 10 ml of 10 mM citrate buffer, pH 6.0. Whole filaments in the cell suspension were fragmented to mostly single cells or fragments containing 2-3 cells, using a Heat System W-385 sonicator. These cells were then treated with NTG (360 μg/ml) for 15 min at room temperature in the light. After washing three times with ASP-2 medium supplemented with 10 mM NaNO₃ (+N medium), the cells were transferred to +N medium and grown in an atmosphere of 1% CO₂-air until the bleached appearance of the cells recovered to a deep blue-green color (about 3 days). Whole filaments were fragmented once again by sonication and then grown in +N medium until the culture reached mid-log phase. The cultures were then transferred to fresh ASP-2 medium and grown for two days. Non-growing cells in a 1% CO₂-air atmosphere were enriched by adding ampicillin (200 μg/ml) to the cultures. After 2 days treatment of ampicillin in ASP-2 medium, the survivors were transferred to +N medium and grown in 1% CO₂-air until they recovered. The ampicillin enrichment procedures were repeated three times; the surviving filaments were sonicated to mostly single cells as before and plated onto ASP-2 plates. Yellow colonies were picked and then restreaked onto fresh ASP-2 plates. Colonies that could grow only under microaerobic conditions, and not under aerobic conditions, were then selected.

Assay of nitrogenase. The acetylene reduction assay was used to measure nitrogenase activity (16). One ml samples of growing cultures were added to 24 ml tubes sealed with serum stoppers, followed by the injection of 1/10 volume of acetylene. Gas samples (1 ml) were analyzed for their ethylene content at the beginning of the incubation period and 30 min later using a Varian 3300 gas chromatograph (Varian Associates, Inc., California 94089) equipped with a flame ionization detector and Poropak N column. Reaction rates were verified to be linear over the time of the assay.

Sample preparation for polyacrylamide electrophoresis. Cells harvested from 120 ml cultures were suspended in 700 μl of sonication buffer containing 50 mM
Tris-Cl, pH 6.8, 1 mM EDTA, 14 mM 2-mercaptoethanol (2-ME). Cells were disrupted using a Heat system W-385 sonicator (Heat Systems-Ultrasonics, Inc. Farmingdale, NY 11735). DNase and RNase were then added to the cell extract to a final concentration of 20 mg/ml and 1 mg/ml, respectively. The samples were kept on ice for 20 min. One volume of 20% TCA in acetone was then added and after 30 min precipitation on ice, proteins precipitates were washed three times with ice-cold acetone. Proteins were then solubilized in in 400 µl lysis buffer containing 9.5 M urea, 5% 2-ME, 2% NP-40, 2% Ampholytes (1.6% Biolytes pH 5-8, 0.4 % Biolytes pH 3-10, BioRad, Richmond Calif. 11.5 cm isoelectrofocusing (IEF) gels were prepared (88,89) and 50 µl samples were loaded on each IEF gel and electrophoresed at 800 V for 16 h. 11.5% SDS polyacrylamide gels, prepared by the protocol of Laemmli (62) were used for the second dimension. Following electrophoresis, gels were stained with Comassie blue or silver-stained using previously described methods (3,62,70).

**Immunoblot (Western) identification of proteins.** Fractionated proteins from 2-dimensional gels were electroblotted onto nitrocellulose filters (Millipore Corp., Bedford, MA) in 25 mM Tris-Cl (pH 8.3)-192 mM glycine-20% methanol with a Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, 1414 Harbor Way South, Richmond, CA 94804). Antiserum against the Fe protein was used to identify the Fe protein of nitrogenase, as previously described (115).

**RESULTS**

**Isolation and growth characteristics of O2 sensitive mutants of *Anabaena* sp. strain CA.** In order to study the mechanism by which the recovery of nitrogenase activity is regulated in strain CA, we sought to generate O2-sensitive mutants that might lack the protection/recovery system. Eleven mutants of strain CA were isolated
by NTG mutagenesis, followed by ampicillin enrichment of non-growing cells in air. Among them, two mutants (N401 and N402) were only able to grow in medium supplemented with fixed nitrogen (nitrate). The inability of these mutants to fix nitrogen indicated that a mutation may have occurred that influenced either the normal structure of heterocysts or the expression and function of nif-specific genes. Perhaps, there was even a defect in some other function required for N\textsubscript{2} fixation. These mutants were not examined further. Nine mutants, which grew normally under aerobic conditions when nitrate or ammonia was provided as a nitrogen source in the absence of fixed nitrogen, were found to be extremely sensitive to O\textsubscript{2}. Four of them, N101, N102, N103 and N104, grew poorly under N\textsubscript{2}-fixing conditions in air, exhibiting doubling times that ranged from 16 h to 22 h. The other five mutants were not able to grow aerobically in the absence of nitrate and ammonia. However, these five mutants grew at varying rates under microaerobic N\textsubscript{2}-fixing conditions, i.e. in a 1% CO\textsubscript{2}/99% N\textsubscript{2} atmosphere (Table 3.1).

**Nitrogenase activity.** The nitrogenase activity of four mutants, N101, N102, N201 and N202, that grew normally in 1% CO\textsubscript{2}/99% N\textsubscript{2}, was compared with wild-type strain CA. When assayed in an atmosphere of 10% C\textsubscript{2}H\textsubscript{2}/air, no nitrogenase activity was detected in mutants N101, N102 and N201, and only 9% of the wild-type level of activity was obtained for strain N202 (Table 3.2). However, when assayed under 10% C\textsubscript{2}H\textsubscript{2}/argon, nitrogenase activity were obtained for all four mutants, ranging from about 29 to 64 percent of the activity found for the wild type.

To test the ability of these mutants to recover from O\textsubscript{2}-mediated inactivation, nitrogenase activity was measured in mutants N101, N102, N201 and N202, grown initially in 1% CO\textsubscript{2}/99% N\textsubscript{2}, and then incubated in 1% CO\textsubscript{2}/99% O\textsubscript{2} from 1 to 3 h. When nitrogenase activity was assayed under 10% C\textsubscript{2}H\textsubscript{2}/argon, strains N201 and N202 showed 16.7% and 30.2% recovery, respectively after 2 h in O\textsubscript{2}, while strains N101 and N102 showed very low recovery (2.2% activity) or no recovery of nitrogenase activity.
Table 3.1. Growth of wild type CA and O$_2$-sensitive mutants in 1% CO$_2$/99% N$_2$ and 1% CO$_2$/air.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling Time (h)</th>
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<tbody>
<tr>
<td></td>
<td>1% CO$_2$/99% N$_2$</td>
</tr>
<tr>
<td></td>
<td>4.6</td>
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</tbody>
</table>

$^a$NG, no growth.
Table 3.2. Nitrogenase activity of wild type strain CA and O$_2$ sensitive mutants grown in 1% CO$_2$/99% N$_2$.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nitrogenase activity ( mmol C$_2$H$_4$/mg dry wt/h )</th>
<th>10% C$_2$H$_2$/argon</th>
<th>10% C$_2$H$_2$/air</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.4 (100%)</td>
<td>1.1 (100%)</td>
<td></td>
</tr>
<tr>
<td>N101</td>
<td>0.9 (64.3%)</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>N102</td>
<td>0.8 (57.1%)</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>N201</td>
<td>0.6 (42.9%)</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>N202</td>
<td>0.4 (28.6%)</td>
<td>0.1 (9.0%)</td>
<td></td>
</tr>
</tbody>
</table>
respectively, after 3 h in O\textsubscript{2} (Table 3.3). However, substantial recovery of activity (62.2\%) was obtained for the wild-type after 3 h in the hyperbaric oxygen atmosphere. The recovery of nitrogenase activity in wild-type strain CA was repressed by the addition of chloramphenicol (0.33\% activity recovered) (data not shown), supporting previous findings which indicated that \textit{de novo} protein synthesis was required for recovery (93,115,116,125). Furthermore, cellular mechanisms involved with the protection of nitrogenase from the deleterious effects of oxygen and the recovery of nitrogenase activity from O\textsubscript{2}-mediated inactivation, might be completely different since mutants N201 and N202 lost their ability to protect nitrogenase from O\textsubscript{2} (Table 3.2) yet still were capable of recovering substantial amounts of activity. Mutants N101 and N102, on the other hand, appeared to have lost both the ability to protect nitrogenase from oxygen and the ability to recover from O\textsubscript{2}-mediated inactivation. These mutants will all be very useful for further studies of the mechanisms involved in the protection and recovery of nitrogenase from oxygen. In the present study, mutant N102 was chosen for further study since its nitrogenase never recovered from oxygen-mediated inactivation.

**Comparison of the protein profiles of wild type strain CA and mutant strain N102 by two-dimensional gel electrophoresis.** To identify proteins that might be involved in the protection and recovery of nitrogenase activity from O\textsubscript{2}-mediated inactivation, two-dimensional gel electrophoresis was performed. The total protein profiles of wild-type strain CA and mutant strain N102 grown under 1\% CO\textsubscript{2}/99\% N\textsubscript{2}, followed by transfer to 1\% CO\textsubscript{2}/99\% O\textsubscript{2} for 3 h, were compared in order to identify proteins whose levels may have changed after transfer. The total protein profiles of cells grown in 1\% CO\textsubscript{2}/99\% N\textsubscript{2} were used as a control. Since the addition of chloramphenicol (CAM) to the growth medium blocked the recovery of nitrogenase activity in O\textsubscript{2}-treated cells, the total
Table 3.3. Recovery of nitrogenase activity by O₂-sensitive mutants of *Anabaena* sp. strain CA.

<table>
<thead>
<tr>
<th>Transitiona</th>
<th>Nitrogenase Activity (mmol C₂H₄/mg dry wt/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N101</td>
</tr>
<tr>
<td>1% CO₂/99% N₂</td>
<td>0.93</td>
</tr>
<tr>
<td>1% CO₂/99% O₂ 1 h</td>
<td>0.01</td>
</tr>
<tr>
<td>1% CO₂/99% O₂ 2 h</td>
<td>-----</td>
</tr>
<tr>
<td>1% CO₂/99% O₂ 3 h</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*aCells were initially grown in a 1% CO₂/99% N₂ atmosphere and switched to an atmosphere of 1% CO₂/99% O₂.*

b*Not determined.*
protein profiles of wild-type strain CA grown in the medium with or without the addition of chloramphenicol was also compared (Figs. 3.1, 3.2).

A total of 15 proteins were identified which show significant changes under the different conditions in both wild-type strain CA and strain N102. Five of these proteins were abundant proteins and were visualized by Coomassie blue staining (C1 - C5) (Fig. 3.1). However, the other ten proteins were less abundant and were detected only after silver staining (S1 - S10) (Fig. 3.2). Based on the patterns of the changes observed under the different conditions (A - E of Figs. 3.1 and 3.2), these 15 proteins may be divided into three groups. Group 1 consists of three proteins: the abundant C4 and C5 proteins (Table 3.4A), and a less abundant protein S4 (Table 3.4B). The level of these three proteins did not increase after 3 h O2 treatment in wild-type strain CA (Table 4); indeed there was a decrease in the relative amount of these proteins, implying that C4, C5 and S4 may not be directly involved in the recovery of nitrogenase activity from O2-inactivation since the recovery required de novo protein synthesis. However, all three proteins showed significantly different levels in the wild-type compared to strain N102 when both were grown under an atmosphere of 1% CO2/99% N2. Proteins C5 and S4 were present in much smaller amounts in strain N102. In contrast to C5 and S4, the C4 protein, a 26.5 kD protein, is present in strain N102 at greater than four times the level found in wild-type strain CA. The significant differences of these proteins in strain CA and N102 suggested that these proteins might possibly be involved in the protection of nitrogenase from the deleterious effects of O2. Both C5 and S4 may be positive factors, and C4 perhaps is a negative factor, that plays a role in the protection process. Subsequent N-terminal sequence analysis of the C5 protein showed identity to the 50 S ribosomal protein L21 of the liverwort Marchantia polymorpha (Fig. 3.3).
Fig. 3.1. Two-dimensional gel analysis of total cellular proteins stained with Coomassie blue of *Anabaena* CA wild-type and O₂-sensitive mutant N102 grown in 1% CO₂/99% N₂. Wild-type grown in 1% CO₂/99% N₂ with no further treatment (A); wild-type cultures switched to 1% CO₂/99% O₂ for 3 h in the absence (B) or presence (C) of chloramphenicol (CAM) just before O₂ treatment; mutant strain N102 grown in 1% CO₂/99% N₂ with no further treatment (D); mutant N102 switched to 1% CO₂/99% O₂ for 3 h (E). Proteins that showed changes in wild-type and in mutant N102 were labeled by numbers (1-5). The amount of these proteins (C1 - C5) on the gels was analyzed using a MicroScan 1000 Gel Analyzer (Technology Resources, Inc., Nashville, Tennessee). Comparison of the relative amount of these proteins (C1 - C5) is shown in Table 4A.
Fig. 3.2. Two-dimensional gel analysis of total cellular proteins (silver stained) of wild-type strain CA and oxygen sensitive mutant N102 grown in 1% CO$_2$/99% N$_2$. Wild-type grown in 1% CO$_2$/99% N$_2$ with no further treatment (A); wild-type cultures switched to 1% CO$_2$/99% O$_2$ for 3 h (B); wild-type cultures switched to 1% CO$_2$/99% O$_2$ for 3 h with chloraphenicol (CAM) added just before O$_2$ treatment (C); mutant N102 grown in 1% CO$_2$/99% N$_2$ with no further treatment (D); mutant N102 cultures switched to 1% CO$_2$/99% O$_2$ for 3 h (E). Proteins that showed changes in wild-type strain CA and mutant N102 grown under different conditions were labeled by numbers (1-10). The amount of these proteins (S1 - S10) on the gels was analyzed using a MicroScan 1000 Gel Analyzer (Technology Resources, Inc., Nashville, Tennessee). Comparison of the relative amount of these proteins (S1 - S10) is shown in Table 4B.
Table 3.4. Comparison of protein profiles of wild type *Anabaena* CA and mutant strain N102.

A. Relative amount of proteins on Coomassie blue stained 2-D gels.\(^a\)

<table>
<thead>
<tr>
<th>Transition (^b)</th>
<th>C1 (59 kd)</th>
<th>C2 (36 kd)</th>
<th>C3 (38 kd)</th>
<th>C4 (26.5 kd)</th>
<th>C5 (18 kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>strain CA, N2 (A)</td>
<td>1.0</td>
<td>1.0</td>
<td>ND(^c)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>strain CA, O(_2) 3h (B)</td>
<td>1.0</td>
<td>1.5</td>
<td>+(^d)</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>strain CA, O(_2) 3h (C) + CAM</td>
<td>0.1</td>
<td>0.1</td>
<td>+</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>strain N102, N2 (D)</td>
<td>ND</td>
<td>0.3</td>
<td>1.2</td>
<td>4.4</td>
<td>+</td>
</tr>
<tr>
<td>strain N102, O(_2) 3h (E)</td>
<td>ND</td>
<td>ND</td>
<td>0.3</td>
<td>2.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\(^a\) The amount of these proteins on gels was analyzed using a MicroScan 1000 Gel Analyzer.
\(^b\) Letters in parentheses refer to panels in Figs. 1 and 2.
\(^c\) ND, not detected.
\(^d\) + present, but not able to be quantitated.

B. Relative amount of proteins on silver stained 2-D gels.

<table>
<thead>
<tr>
<th>Transition</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
<th>S9</th>
<th>S10</th>
</tr>
</thead>
<tbody>
<tr>
<td>strain CA, N2 (A)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>strain CA, O(_2) 3h (B)</td>
<td>1.1</td>
<td>3.6</td>
<td>6.0</td>
<td>0.21</td>
<td>8.9</td>
<td>2.1</td>
<td>1.0</td>
<td>1.5</td>
<td>27.8</td>
<td>2.1</td>
</tr>
<tr>
<td>strain CA, O(_2) 3h (C) + CAM</td>
<td>ND</td>
<td>ND</td>
<td>1.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.9</td>
<td>0.7</td>
</tr>
<tr>
<td>strain N102, N2 (D)</td>
<td>0.4</td>
<td>ND</td>
<td>0.3</td>
<td>ND</td>
<td>3.6</td>
<td>0.9</td>
<td>0.4</td>
<td>1.1</td>
<td>1.2</td>
<td>0.6</td>
</tr>
<tr>
<td>strain N102, O(_2) 3h (E)</td>
<td>0.1</td>
<td>ND</td>
<td>0.2</td>
<td>ND</td>
<td>3.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Fig. 3.3. Comparison of the N-terminal amino acid sequences of the 50S ribosomal L21 protein of *Marchantia polymorpha*, and the C5 protein of *Anabaena* sp. strain CA. The N-terminal sequence of the C5 protein showed 90.5% identity to the L21 protein in a 21 amino acid overlap region.
Group 2 consists of seven proteins, including three abundant proteins, C1, C2 and C3, and four less abundant proteins, S2, S5, S7, and S8. All these proteins show either a constant level, or an increased level in wild-type CA after 3 h treatment with O2. In strain N102, these proteins either showed a decreased level or were completely absent after 3 h O2 treatment (Table 3.4). In addition, the presence of chloramphenicol during O2 treatment resulted in a significant decrease in the amount of all these proteins in wild-type strain CA, except for C3, which appeared to be missing in cultures incubated with N2. However, after 3 h in O2, the C3 protein was induced in wild-type strain CA. These results indicated that proteins C1, S5, S7, and S8 may be potential candidates involved in the recovery of nitrogenase from O2-mediated inactivation. The unusual property of the C3 protein after treatment with chloramphenicol is discussed below.

Group 3 includes five less abundant proteins; S1, S3, S6, S9 and S10. The level of these proteins increased to various degrees in wild-type strain CA after 3 h treatment with O2, but similar levels were maintained, or even decreased in amount, after 3 h of O2 treatment in strain N102. Furthermore, the addition of chloramphenicol during the O2 treatment caused a decrease in the amount of these proteins in wild-type CA. These results suggested that proteins S1, S3, S6, S9 and S10 may be involved in the recovery of the nitrogenase activity after O2 treatment.

To determine whether any of these fifteen proteins was dinitrogenase reductase (the Fe protein or component II) of the nitrogenase complex, antiserum against the Fe protein from *Rhodospirillum rubrum* was used to probe Western blots. Two abundant proteins: C2, with a size of 36 kD, and C3, with a size of 38 kD were identified as the Fe protein (data not shown). In wild-type strain CA, both the 36 kD and the 38 kD Fe protein increased in amount after O2 treatment (Table 3.4A). However, a significant decrease in both the 36 kD and 38 kD proteins was found in strain N102; in particular, the 36 kD protein totally disappeared in strain N102 after O2 treatment. In wild-type strain CA, the
addition of chloramphenicol during the O\textsubscript{2} treatment resulted in a drastic decrease in the amount of the 36 kD protein but a significant increase in the amount of the 38 kD protein. This result was consistent with previous observations which showed that the 38 kD protein is a modified form of the 36 kD protein; the presence of chloramphenicol was found not to prevent the modification (125).

**DISCUSSION**

To identify potential protein components of the recovery/inactivation process, we isolated new oxygen-sensitive mutants in *Anabaena* sp. strain CA. Nine oxygen-sensitive mutants were isolated by NTG mutagenesis. These mutants showed different levels of O\textsubscript{2} sensitivity. They grew poorly or not at all under nitrogen-fixing conditions aerobically, but all the strains grew normally when fixed nitrogen (ammonia or nitrate) was added to the growth medium. These results exclude the possibility that the nitrate and ammonium assimilatory mechanisms were damaged in these mutants.

The nitrogenase activity of four mutants, N101, N102, N201, and N202 was comparable to wild-type strain CA when assayed under microaerobic conditions (10% C\textsubscript{2}H\textsubscript{2}/argon). When assayed under aerobic conditions (10% C\textsubscript{2}H\textsubscript{2}/air), no nitrogenase activity was detected in strain N101, N102 and N201, and only 9% of the wild-type level was obtained in strain N202 (Table 3.2). These results indicated that all these mutants, especially N101, N102 and N201, may have lost their ability to protect nitrogenase from O\textsubscript{2} injury.

By comparing the protein profiles of wild-type strain CA and oxygen-sensitive mutant strain N102 grown under different conditions, a total of fifteen proteins, five abundant proteins (C1 - C5) and ten less abundant proteins (S1 - S10), were found to exhibit significant changes in both strains during O\textsubscript{2} treatment. The C2 and C3 proteins
were identified as the unmodified 36 kD and the modified 38 kD Fe protein of nitrogenase, respectively. In wild-type strain CA, the level of both the 36 kD and 38 kD Fe protein forms increased after the whole filaments were exposed to hyperbaric O2 when compared to cells grown in 1% CO2/99% N2. In addition, the presence of chloramphenical during the O2 treatment resulted in a significant increase in the amount of the 38 kD inactive form of the Fe protein and a parallel loss of the 36 kD (active) form of the Fe protein. These results support previous observations that nitrogenase synthesis is not repressed by exposure to O2, and the recovery of activity is not simply due to de novo synthesis of nitrogenase in Anabaena sp. strain CA. Other newly synthesized proteins may be involved in the recovery process and may be involved in either the activation of the 38 kD Fe protein or in the protection of the 36 kD protein from inactivation. In many diazotrophic bacteria, nitrogenase synthesis is repressed in the presence of O2. This strategy prevents wasteful synthesis of inactive nitrogenase. However, in some cyanobacteria, nitrogenase synthesis is not repressed by oxygen as shown in Anabaena variabilis (84), and the unicellular organism Gloeothecae (74). It has also been shown in Anabaena cylindrica that the turnover of nitrogenase is stimulated by O2 (85). Thus, in strain CA, newly synthesized proteins obviously contribute to the nitrogenase activity found in the presence of oxygen. However, the addition of chloramphenicol during O2 treatment resulted in a significant increase in amount of the 38 kD inactive form of the Fe protein, suggesting that newly synthesized proteins during recovery are either involved in the activation of the 38 kD protein or in the protection of the 36 kD active form of the Fe protein from inactivation by O2. It has also been reported that alternative nitrogenases are present in a variety of cyanobacteria (127) and an alternative nitrogenase was shown to be expressed in vegetative cells of A. variabilis (128). Since we used whole filaments of wild-type Anabaena sp. strain CA and mutant strain N102 that were initially grown in 1% CO2/99% N2 for 2 D gel electrophoreses, an alternative nitrogenase that contributes to the recovery of activity could
possibly have been induced in undifferentiated vegetative cells under these microaerobic conditions. Comparing the levels of the 36 kD protein in wild-type strain CA and mutant strain N102 with the level of nitrogenase activity suggested that the inability of strain N102 to recover nitrogenase activity after O2 treatment might perhaps result from the very rapid degradation of nitrogenase or the failure of the 36 kD protein to be synthesized de novo during O2 treatment. A C5 protein was found to be present in much smaller amounts in strain N102 than in wild-type. Subsequent N-terminal sequence analysis of the C5 protein showed identity to the 50S ribosomal protein L21 of the liverwort Marchantia polymorpha. Since O2 treatment might increase the turnover rate of some proteins in response to O2 stress, wild-type strain CA and the mutant strain might perhaps need to increase the level of protein synthesis to maintain full enzyme activity of nitrogenase or other proteins involved in protection. Since the nitrogenase of strain N102 was not able to become protected or recover from O2-mediated inactivation, one of the reasons might be due to some defect in the synthesis of ribosomal protein L21, which may be symptomatic of some inability of this mutant to synthesize proteins during hyperbaric oxygen stress. Further studies are obviously required to test this hypothesis, however the identification of protein L21, and presumably other proteins in future work, should enable specific mutants to be constructed in Anabaena species.

ACKNOWLEDGEMENTS

We thank the Ohio State Biochemical Instrumentation Center for N-terminal analyses.
CHAPTER IV

Transcription of Genes Involved in Polysaccharide Biosynthesis in *Anabaena* sp. Strain CA Appears to be Implicated in Functional Nitrogenase Activity Under Hyperbaric Oxygen Concentrations

INTRODUCTION

Like many other nitrogen-fixing organisms, the nitrogenase enzymes of cyanobacteria are highly sensitive to oxygen. Since cyanobacteria perform oxygenic photosynthesis, the oxygen produced from photosynthesis is potentially a problem for the proper functioning of the nitrogenase complex in these organisms. As is the case in most microbial processes, aerobic cyanobacteria have evolved a variety of mechanisms to prevent nitrogenase from being inactivated by oxygen, such as temporal regulation, high rates of O$_2$ consumption and respiration in unicellular and nonheterocystous strains, plus a unique spatial separation of the processes of photosynthesis in vegetative cells and nitrogen fixation in heterocysts, respectively, in filamentous differentiating cyanobacteria (18,28,36,44,79,95,120,121). Mature heterocysts lack photosystem II and have a thick cell envelope that consists of glycolipids and polysaccharides that are believed to serve as partial O$_2$ diffusion barriers since mutants of *Anabaena* 7120 with aberrant heterocyst envelope glycolipids failed to express active nitrogenase under aerobic conditions (86). However, it is apparent that this is not the full story since the same laboratory has isolated oxygen-sensitive mutants that are not altered in their heterocyst glycolipid content (49).

The nitrogenase from several *Anabaena* spp. was found to recover from O$_2$-mediated inactivation (93). In *Anabaena* sp. strain CA, previous studies had shown that the initial
inactivation of nitrogenase activity under an atmosphere of 1% CO₂/99% O₂ was followed by a recovery phase. It was also found that hyperbaric O₂ treatment result in a decrease in the mobility of dinitrogenase reductase. The function of this modification is unclear but it may render nitrogenase more oxygen tolerant (93, 115, 125). The recovery of activity by strain CA involved de novo proteins synthesis, and recovery of activity was repressed by ammonia (93, 116), an interesting finding since ammonia only partially represses nitrogenase synthesis in this organism. To identify potential protein components that might be important for the protection and/or recovery of nitrogenase from O₂-mediated inactivation in *Anabaena* CA, several approaches have been attempted. By comparing the 2-D gel electrophoretic protein profiles of wild-type strain CA and O₂-sensitive strain N102, several proteins that might be involved in the recovery of nitrogenase activity were identified. However, their abundance is not great, making it difficult to isolate and identify the genes which encode these proteins (20).

A genetic screen is the traditional method employed to identify genes involved in complicated biological processes. In particular, subtractive hybridization methods may be effectively used to identify mRNAs that differ in abundance between two distinct mRNA populations. This strategy has been successfully applied, for example, to identify sporulation specific genes, virulence genes, and sugar metabolism regulatory genes from a number of bacteria, as well as salt-induced genes of the cyanobacterium *Anabaena torulosa*, and a light repressed transcript of *Synechococcus* sp. strain PCC 7002 (1, 60, 75, 102, 126).

Since the previous studies in *Anabaena* CA had found that recovery of nitrogenase activity required protein synthesis, we sought to use subtractive hybridization approaches to isolate genes of *Anabaena* CA that are differentially expressed in hyperbaric O₂ that might be important for the recovery/protection of nitrogenase from O₂-mediated inactivation. In this study, we report the isolation and characterization of genes of *Anabaena* CA, identified
from subtractive hybridization whose sequence suggests might be involved in polysaccharide synthesis and aerobic nitrogen fixation.

**MATERIALS & METHODS**

**Strain and culture conditions.** *Anabaena* sp. strain CA (ATCC 33047) was grown photoautotrophically in ASP-2 medium (NaCl, 5 g/l) at 39°C at constant illumination and bubbled with 1% CO2/99% N2 with or without 1% CO2/99% O2 treatment (115,131).

**RNA extraction and Northern blot analysis.** Total RNA was extracted from mid-exponential growing cultures of *Anabaena* sp. strain CA grown in 100 ml ASP-2 medium in the presence of combined nitrogen (10 mM NaNO3), with or without 1% CO2/99% O2 treatment. RNA was extracted and analyzed as described previously (53). RNA (10 μg of each sample) was fractionated on formaldehyde gels and then transferred onto Gene Screen Plus membranes (DuPont, Boston, MA). Hybridizations were carried out in aqueous solutions containing 10% dextran sulfate, 1% SDS, 1 M NaCl, based on instructions provided by the manufacturer. The size of the transcripts obtained was determined from the migration in gels relative to an RNA ladder (BRL, Gaithersburg, MD). Northern blots were hybridized with random-primed-labeled probes (30). Probes for hybridizations were derived from the 3.7 kb *HindIII* fragment (fragment 31-2), 0.7 kb *StsI-MunI* fragment (ORF 1), 0.47 kb *BstXI-BstYI* fragment (ORF 2), and 0.83 kb *EcoRI-HindIII* fragment (ORF 3) of *Anabaena* sp. strain CA DNA (Fig. 4.3).

**cDNA synthesis.** Total RNA isolated from *Anabaena* sp. strain CA initially grown in 1% CO2/99% N2 and then transferred to 1% CO2/99% O2 for 2 h was used for cDNA synthesis. [32P] labeled cDNA was synthesized using 5 μg of *Anabaena* CA RNA as template in a 50 μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 6 mM
MgCl₂, 1 mM dATP, 1 mM dGTP, 50 μM dCTP, 1 mM dTTP, 50 μCi of [α-³²P]dCTP (3000 Ci/mmol), random primers (200 μg/ml, Pharmacia, Piscataway, NJ), 30 mM dithiothreitol, 100 units of RNasin, and 50 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, IN). After 2 h incubation at 42°C, an equal volume of 0.2 N NaOH was added to the mixture and the mixture was incubated at 70°C for 30 min to hydrolyze the RNA.

**Preparation of subtracted cDNA probes.** After RNA hydrolysis, [³²P]cDNA was ethanol precipitated with 100 μg of total RNA from *Anabaena* CA grown in 1% CO₂/99% N₂. The precipitates, containing cDNA and RNA, were then resuspended in a 50 μl hybridization solution (0.5 M phosphate buffer, pH 6.9, 5 mM EDTA, 0.1% SDS), and briefly heated to 80°C. The hybridization was carried out at 68°C for 24 h (75). All double-stranded cDNA-RNA hybrids were selectively removed and the single stranded [³²P]cDNA was eluted by hydroxylapatite chromatography at 60°C. Fractions were found to contain predominantly single stranded [³²P]cDNA and were pooled and subsequently used to probe a genomic library of *Anabaena* CA (Fig. 4.1) (67).

**Genomic library screening.** An O₂-induced, subtracted cDNA probe from *Anabaena* CA was used to screen the genomic library of *Anabaena* CA (67). Colony hybridization was performed based on the procedures described by Maniatis et al. (72).

**Southern hybridization of selected clones using cDNA probes prepared from RNA of *Anabaena* CA.** Plasmid DNA prepared from positive clones was digested with *Hind*III, electrophoresed on 0.8% agarose gels, and then blotted onto Gene Screen Plus membrane according to protocols described by the manufacturer. DNA blots were hybridized with [³²P]cDNA probes prepared from RNA of *Anabaena* CA grown in 1% CO₂/99% N₂ (N₂ cDNA probe) or RNA from strain CA initially grown in 1% CO₂/99% N₂ and then transferred to 1% CO₂/99% O₂ for 2 h (O₂ cDNA probe). Restriction fragments that showed higher hybridization signals with the O₂ cDNA probe
Fig. 4.1. Strategy for cloning O2-induced genes. For preparing a subtracted O2 cDNA probe, total RNA was purified from cultures of Anabaena sp. strain CA that were initially grown in an atmosphere of 1% CO2/99% N2 and then transferred to an atmosphere of 1% CO2/99% O2 for 2 h. [32P]cDNA was synthesized, using 5 μg total RNA from O2 treated cultures of Anabaena CA, with AMV reverse transcriptase and random hexanucleotide primers. After alkaline hydrolysis, [32P]cDNA was hybridized in solution with 80 μg total RNA from Anabaena CA cultures grown in an atmosphere of 1% CO2/99% N2. The single stranded [32P]cDNA eluted by hydroxylapatite chromatography at 60°C was used to screen a genomic library of Anabaena CA.
$N_2 \rightarrow O_2$ for 2 h, isolate *Anabaena* CA RNA

reverse transcription
alkaline hydrolysis

$O_2^{[32-P]}$ cDNA

hybridization
excess $N_2$ RNA

RNA/cDNA hybrids

RNA
cDNA

hydroxylapatite chromatography

$O_2^{[32-P]}$ cDNA
$N_2$ RNA

alkaline hydrolysis

$O_2^{[32-P]}$ cDNA probe

library screening

detection of differentially expressed genes by autoradiography

Fig. 4.1.
than with the N\textsubscript{2} cDNA probe were subcloned into pBluescript SK+ for further investigation.

**Sequence analysis of the 3.7 kb *HindIII* fragment (31-2) identified from subtractive hybridization.** A 3.7 kb *HindIII* fragment, fragment 31-2 identified from subtractive hybridization, was subcloned into pBluescript SK+ in both orientations. Two sets of nested deletion clones were generated for sequencing using protocols described by the Promega (Madison, Wis.) Protocols and Applications Guide. The dideoxy-chain termination sequencing method (109) was used to sequence double-stranded DNA templates, using the U.S. Biochemical sequenase version 2.0 kit, M13/pUC forward 23-base sequencing primer. Six oligonucleotides synthesized by Bio-Synthesis, Inc. (Lewisville, Tex.) were also used as primers to complete the sequence. Sequence data were analyzed using the sequence analysis programs of the University of Wisconsin Genetics Computing Group (GCG) and also by MacVector Sequence analysis Software of International Biotechnologies, Inc. (New Haven, Conn.).

**Primer extension analysis.** Primer extension analysis was performed using the methods described previously (4). A 20-mer oligonucleotide (5'-AAGTATGAACCATCTTGGCC-3'), complementary to the +40 to +59 region with respect to the translational start of ORF 2, was end labeled with $[^{32}\text{P}]ATP$. About $10^6$ cpm of labeled primers was mixed with 100 μg of *Anabaena* sp. strain CA RNA, isolated from a culture grown initially in 1% CO\textsubscript{2}/99% N\textsubscript{2} and then switched to 1% CO\textsubscript{2}/99% O\textsubscript{2} for 2 h. To identify the transcriptional start sites of ORF 2, the same primer was used to synthesize a sequence ladder from plasmid pAC31-2A.
RESULTS

Screening for transcripts induced in hyperbaric O\textsubscript{2}. To identify genes of *Anabaena CA* whose expression is regulated by hyperbaric O\textsubscript{2} treatment, we developed a strategy using a subtracted O\textsubscript{2} cDNA probe to screen a genomic library of strain CA (Fig. 4.1). Upon screening 3400 clones of the cosmid library of strain CA with the subtracted probe (Fig. 1), a total 169 clones were found to hybridize. These clones were purified and characterized further.

Southern blot analysis. Duplicate blots of recombinant cosmid DNAs digested with *HindIII* were hybridized with equal amounts of [\textsuperscript{32}\text{P}]cDNA to total RNA extracted from cells grown in 1% CO\textsubscript{2}/99% N\textsubscript{2} as well as total RNA extracted from *Anabaena CA* initially grew in 1% CO\textsubscript{2}/99% N\textsubscript{2} and then transferred to 1% CO\textsubscript{2}/99% O\textsubscript{2} for 2 h. Southern hybridization identified several clones containing fragments that yielded a stronger signal from the probes prepared from RNA of *Anabaena CA* cells incubated with hyperbaric O\textsubscript{2} than for RNA extracted from cells incubated in the absence of O\textsubscript{2} treatment (data not shown). These results suggested that the isolated clones contained genes which had higher expression in O\textsubscript{2} than in N\textsubscript{2}. Subsequent Southern hybridization, using a plasmid containing the 5 S, 16 S, and 23 S rRNA genes to probe *HindIII* digested DNA purified from these cosmid clones, indicated that most of the clones contained rRNA sequences (129). These clones were not examined further. However, fragment 31-2 from clone 31 proved interesting.

Increase of fragment 31-2 specific mRNA during O\textsubscript{2} treatment. The 3.7 kb *HindIII* of fragment 31-2, presumably containing O\textsubscript{2}-regulated sequences, was used to probe Northern blots prepared from RNA of *Anabaena CA* grown in 1% CO\textsubscript{2}/99% N\textsubscript{2} and also from RNA prepared from cells initially grown in 1% CO\textsubscript{2}/99% N\textsubscript{2} and then transferred to 1% CO\textsubscript{2}/99% O\textsubscript{2} for 2 h. The same blot was stripped and then reprobed with
Fig. 4.2. Northern blot analysis of fragment 31-2 (identified from subtractive hybridization) in *Anabaena* CA. Total RNA (10 μg) was obtained from strain CA cultures grown in 1% CO₂/99% N₂ (N₂) and then switched to 1% CO₂/99% O₂ for 2 h (O₂). A probe specific to fragment 31-2 and a 16 S rRNA gene probe containing the 16 S rRNA gene of *R. spheroides* were used to quantify the amount of fragment 31-2 specific mRNA and 16 S rRNA transcripts in the same blot. The level of transcription was quantitated using a Betagen analyzer.
Fig. 4.2.

16 S rRNA probe

N₂ O₂

frag. 31-2 probe

N₂ O₂

--- 2.2 kb
a 16 S rRNA probe of *R. sphaeroides*. The results of this experiment indicated that the levels of the 16 S rRNA gene transcript was the same under both growth conditions (determined with a Betascope analyzer). While a 2.2-kb transcript was found in the blot probed with fragment 31-2, this transcript increased about 5-fold after 2 h incubation in 1% CO2/99% O2. The 3.7 kb *HindIII* fragment was subsequently subcloned into pBluescript SK+ in both orientations, designated pAC31-2A and pAC31-2B, respectively.

**Sequence analysis of the entire 31-2 fragment and identification of open reading frames.** Northern blot analysis with the 31-2 fragment probe indicated there was a 5-fold increase in transcription in *Anabaena* strain CA after 2 h O2 treatment. To identify the O2-regulated genes in fragment 31-2, the sequence of both strands of this 3.7 kb *HindIII* fragment was determined (see Fig. 4.3 for a summary). The size of this fragment is 3675 bp and contains two complete ORFs and two partial ORFs sequences (Figs. 4.3, 4.4). Fig. 4 presents the nucleotide sequence of the entire 3675 bp *HindIII* fragment and the predicted amino acid sequence of the encoded ORFs of this fragment (ORFs 1-4). ORF 1 begins at nucleotide 376 and continues to nucleotide 1140; it has the potential to encode a protein with a predicted mass of 29,309 Da. There is a potential ribosome-binding site (5'-GGATTAA-3') that precedes the ORF 1 translation start by 8 bp. The putative -10 and -35 region of ORF 1, 5'-TGTAAT-3' and 5'-TTGGAA-3', precedes the ORF 1 translation start by 64 bp and 89 bp, respectively (Fig. 4.5). ORF 2 is found downstream of ORF1 and is separated from ORF1 by an intergenic region of 573 bp. This ORF has the potential to encode a protein with a deduced mass of 41,045 Da (Figs. 4.3 and 4.4). A 62 bp direct repeat is found in the intergenic region of ORF 1 and ORF 2 (Fig. 4.4). These sequence may have some regulatory function in transcription and may act to either terminate transcription or stabilize the mRNA. The potential ribosome-binding site 5'-AAAGGA-3' precedes the ORF 2 translation start by 11 bp. The putative -10 and -35 regions, 5'-TATATT3' and 5'-TTGACT-3', precede the ORF 2 translation start by 130
Fig. 4.3. Restriction map of fragment 31-2 of *Anabaena* sp. strain CA. The solid arrows below the map indicate the direction and length of sequencing. The position of ORFs 1, 2, 3, and 4 in this fragment are indicated. The thick arrows above the map indicate the transcription orientation. Not all restriction sites are indicated on the map. H, *HindIII*; S, *SstI*; By, *BstYI*; Bx, *BstXI*; E, *EcoRI*; Ev, *EcoRV*; M, *MunI*.
Fig. 4.4. Nucleotide and deduced amino acid sequences of the 3.7-kb HindIII fragment from clone 31-2 identified by subtractive hybridization. Potential ribosome binding sites, putative promoter sequences, and a 62 bp direct repeat between ORF 1 and ORF 2 are underlined. Possible transcriptional start sites of ORF 2 are shown in boldface.
Fig. 4.4
Fig. 4.4. (continued)

(1601) GCCAGCTTTAATAAACCTGTATTTATCCATTAACTACCTAGGTTGTGTAATATTTGACCTCTTCTTTAGGGACAAAGCTGG

(1701) ATAGCCGCTCTACTATGAACACAAAGCTGTTAAGTGATATTATCTAAGCTGCTATTTATGCATCTATATTCTAGCTGCT

(1801) GCTATGTTAATAATCCCAAGCTTACCTTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(1901) ACCTGCTCTTCTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(2001) CTTCGTATTCGCCTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(2101) AGCTCTTACTGCTTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(2201) CCAAGCTTACTGCTTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(2301) TGAACCTTACTGCTTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(2401) TGGCTCTAAGCTTACTGCTTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(2501) GGGCTCTAAGCTTACTGCTTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(2601) AATGGCTCTAAGCTTACTGCTTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(2701) ATAGCCGCTCTACTATGAACACAAAGCTGTTAAGTGATATTATCTAAGCTGCTATTTATGCATCTATATTCTAGCTGCT

(2801) CCAAGCTTACTGCTTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(2901) GATGCTCTAAGCTTACTGCTTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT

(3001) ATCTCCTCAATGCTTACTGCTTCTTTAGTGACCTCAGTTCATTACGAACTGACCTTCTCTATTTTCTCTTCTCT
and 146 bp, respectively. The partial sequence of ORF 3, found downstream of ORF 2, is separated from ORF 2 by 25 bp. A potential ribosome-binding site 5'-AAGGAT-3' occurs 4 bp upstream of the ORF 3 translation start. An additional partial sequence, designated ORF 4, is found upstream of ORF 1.

**Sequence comparisons.** The nucleotide sequence of the entire 3675 bp fragment revealed two complete and two partial open reading frames (ORFs) (Figs. 4.3, 4.4). All four ORFs are transcribed in the same direction. The deduced amino acid sequence of each of these ORFs was compared with the known protein data base using the Fasta and Blast programs of the GCG package. Bestfit and Pileup programs of the GCG package were used to align the predicted sequences of the ORFs with similar proteins. The predicted amino acid sequence of ORF 1, containing 253 amino acids, showed high identity to the C-terminal halves of the RfbP protein of *Salmonella typhimurium* and the *ams* gene product of *Erwinia amylovora* (13,133,134). This ORF also showed identity to proteins involved in the biosynthesis of polysaccharides in other organisms, including ExoY, CpsD, Pss4, and GumD (55,97,104,138). An alignment of ORF 1 with ExoY and the C-terminal halves of RfbP and Ams are present in Fig. 4.5. A comparison of ORF 1 with RfbP, ExoY, Pss4, CpsD, and GumD and Ams is presented in Table 4.1.

The deduced amino acid sequences of ORF 2 is 359 amino acids in length. A search of the protein data base indicated that ORF 2 showed high identity to proteins involved in the synthesis of lipopolysaccharide in various organisms. Fig. 4.6 presents the alignment of the predicted amino acid sequences of ORF 2 with *Vibrio cholerae* RfbD, and the *E. coli* YefA protein. The deduced amino acid sequence of ORF 2 showed 61% identity and 75% similarity to the RfbD of *V. cholerae*. ORF 2 also showed high identity to GDP-D-mannose dehydratases (YefA) from *E. coli* (62% identity and 77% similarity) and *Pseudomonas aeruginosa* (gca gene product) (52.6% identity and 70.4% similarity) (22,122,138). The 287 amino acid sequences deduced from partial ORF 3 is found to be
Fig. 4.5. Deduced amino acid sequence of ORF1 of fragment 31-2 from *Anabaena* sp. strain CA compared with ExoY of *Rhizobium* sp. strain ngr234 (Exoy1), ExoY of *R. meliloti* (Exoy2), ExoY of *Agrobacterium radiobacter* (Exoy3), RfbP of *Salmonella typhimurium* (RfbP), an RfbP homolog of *Haemophilus influenzae* (Hi), and Ams of *Erwinia amylovora* (Ams). The alignment was determined using the Pileup program of the GCG package. Identical residues are shaded.
Fig. 4.5.
Table 4.1. Comparison of deduced amino acid composition of ORF 1 with other proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>No. of amino acids</th>
<th>% identity</th>
<th>% similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ExoY</td>
<td><em>Rhizobium meliloti</em></td>
<td>226</td>
<td>40.9%</td>
<td>65.8%</td>
</tr>
<tr>
<td>ExoY</td>
<td><em>Agrobacterium radiobacter</em></td>
<td>226</td>
<td>42.2%</td>
<td>65.3%</td>
</tr>
<tr>
<td>CpsD</td>
<td><em>Streptococcus agalactiae</em></td>
<td>274</td>
<td>33.3%</td>
<td>56.1%</td>
</tr>
<tr>
<td>Pss4</td>
<td><em>Rhizobium leguminosarum</em></td>
<td>264</td>
<td>35.9%</td>
<td>58.5%</td>
</tr>
<tr>
<td>RfbP</td>
<td><em>Salmonella typhimurium</em></td>
<td>476</td>
<td>44.4%</td>
<td>65.6%</td>
</tr>
<tr>
<td>Ams</td>
<td><em>Erwinia amylovora</em></td>
<td>477</td>
<td>40.9%</td>
<td>66.0%</td>
</tr>
<tr>
<td>GumD</td>
<td><em>Xanthomonas campestris</em></td>
<td>484</td>
<td>35.7%</td>
<td>57.4%</td>
</tr>
</tbody>
</table>

*The % identity and % similarity were determined using the Bestfit program of the GCG package.*
Fig. 4.6. Comparison of the deduced amino acid sequence of ORF2 of fragment 31-2 from *Anabaena* sp. strain CA to *Vibrio cholerae* RfbD and the GDP-D-mannose dehydratases of *E. coli* (YefA) and *Pseudomonas aeruginosa* (GCA). Residues identical to all three proteins are shaded. The alignment was determined by using the Pileup program of the GCG package. Identical residues are shaded.
Fig. 4.6.
related to *E. coli* YefB, *Azorhizobium* NolK, and *Yersinia enterica* ORF 14.8, proteins which have also been implicated in the synthesis of lipopolysaccharide (37, 76). Another partial ORF, ORF4, is located upstream of ORF 1. The deduced amino acid sequence of ORF 4 indicated that it was related to the *Salmonella enterica* first mannosyl transferase (59).

**O2 effects on the transcription of genes encoding ORF 1, ORF 2, and ORF 3.** In previous studies, when RNA blots were probed with the 31-2 fragment of strain CA, we observed a 5-fold increase in the level of a 2.2 kb transcript after 2 h incubation in an atmosphere of 1% CO2/99% O2. As discussed above, sequence analysis of the entire 3.7 kb 31-2 fragment revealed two complete ORFs and two partial ORFs in this fragment. To determine which ORF(s) might be responsible for the 2.2 kb transcript, and which gene is affected by hyperbaric O2, we employed intergenic probes specific to ORF 1, ORF 2, and ORF 3 to analyze additional Northern blots. Total RNA was extracted from *Anabaena CA* cells grown under nitrogen-fixing conditions bubbled with 1% CO2/99% N2 and also from cells grown initially in 1% CO2/99% N2 and then transferred to 1% CO2/99% O2 for 2 h. Fig. 4.7 presents the results of the Northern hybridization experiments. Probes specific for ORF 2 hybridized to a 2.2 kb transcript, which is present in both 1% CO2/99% N2 grown cells and O2-treated cells. The level of this transcript in *Anabaena CA* increased about 2.1 fold after 2 h of O2 treatment. When probed with ORF 1, although several hybridization experiments were attempted, no transcripts were detected in *Anabaena CA*. When probed with ORF 3, the level of the 2.2 kb transcript also increased in O2 (results not shown). To determine whether this 2.2 kb mRNA was enriched in heterocysts, we examined the effects of O2 on the expression of this mRNA in *Anabaena CA* filaments containing only vegetative cells; i.e. total RNA was extracted from filaments grown in ASP-2 medium supplemented with 10 mM NaNO3 (Fig. 4.8). When probed with an ORF 2 specific probe, the 2.2 kb transcript was present in
Fig. 4.7. Northern blot analysis of ORF 1 and ORF 2 of fragment 31-2 in *Anabaena* CA. Total RNA (10 μg) was obtained from strain CA grown in 1% CO₂/99% N₂ (N₂) and strain CA grown in 1% CO₂/99% N₂ and then transferred to 1% CO₂/99% O₂ for 2 h (O₂). A *SstI-MunI* fragment ORF 1 probe, *BstXI-BstYI* fragment ORF 2 probe, and a 16 S rRNA probe from *R. spheroides* were random prime labeled and used to identify the ORF 1 and ORF 2 specific mRNA, followed by determining the amount of 16 S rRNA transcripts in the same blots. The level of transcription was quantitated with a Betascope 603 blot analyzer (Betagen, Mountain View, Calif.).
Fig. 4.7.
Fig. 4.8. Northern blot analysis of ORF 2 of fragment 31-2 in vegetative cells of *Anabaena* CA. *Anabaena* CA was grown in ASP-2 medium supplemented with 10 mM NaNO₃. Total RNA (10 μg) was obtained from strain CA grown in 1% CO₂/99% N₂ (N₂) or grown in 1% CO₂/99% N₂ and then switched to an atmosphere of 1% CO₂/99% O₂ for 2 h (O₂). A *Bst*XI-*Bst*YI fragment from ORF 2 of clone 31-2 and a 16 S rRNA gene probe from *R. sphaeroides* were random prime labeled and used in the hybridizations. The level of transcription was quantitated using a Betascope 603 blot analyzer (Betagen, Mountain View, Calif.).
Fig. 4.8.
vegetative cells of *Anabaena* CA grown in 1% CO$_2$/99% N$_2$ with or without O$_2$ treatment. Furthermore, a 4-fold decrease of this mRNA was observed in the vegetative cells after 2 h of O$_2$ treatment. These results indicate that in *Anabaena* CA the expression of this 2.2 kb transcript is not heterocyst specific, however the expression of ORF 2 and ORF 3 is significantly decreased in undifferentiated cultures i.e., containing filaments that lack heterocysts.

To investigate the effects of ammonia on the induction of ORF 2 and ORF 3 expression in O$_2$, 10 mM NH$_4$Cl was added to the medium at the beginning of the 2 h O$_2$ incubation (Fig. 4.9). Consistent with previous results, when probed with internal fragments of either ORF 2 or ORF 3, the level of the 2.2 kb transcript increased about four-fold in strain CA after 2 h in the 1% CO$_2$/99% O$_2$ atmosphere compared to cells incubated without the O$_2$ treatment. However, the presence of NH$_4$Cl during O$_2$ treatment repressed the accumulation of this transcript in *Anabaena* CA. The level of this 2.2 kb transcript in strain CA was not induced if NH$_4$Cl was added to the medium prior to the O$_2$ treatment (Fig. 4.9).

Northern blot studies using the ORF 2 and ORF 3 specific probes detected the same 2.2 kb transcript, therefore it would seem that ORF 2 and ORF 3 are cotranscribed. To map the transcription start of ORF 2, a primer extension experiment was performed using RNA from O$_2$ treated cells. Four putative transcription start sites were detected at positions -3, -47, -123, and -157 with respect to the translational start of ORF 2 (Figs. 4.4, 4.10).

**DISCUSSION**

To isolate genes important for the protection/recovery of nitrogenase activity in *Anabaena* sp. strain CA, we sought to use a subtractive hybridization strategy to clone up-regulated genes in the presence of hyperbaric O$_2$ since such genes might be involved in the
Fig. 4.9. The effects of ammonia metabolism on the accumulation of ORF 2 and ORF 3 specific mRNA under hyperbaric O₂ concentrations. Total RNA (10 µg) was obtained from *Anabaena* CA grown in 1% CO₂/99% N₂ (N₂), and then switched to an atmosphere of grown in 1% CO₂/99% O₂ for 2 h in the absence (O₂) or presence of 10 mM NH₄Cl (NH₄Cl). A random primed *Bst*XI-*Bst*YI fragment from ORF 2, an *Eco*RI-*Hind*III fragment from ORF 3, and a 16 S rRNA gene probe were used in the Northern blot hybridizations. The level of transcription was quantitated using a Betascope 603 blot analyzer (Betagen, Mountain View, Calif.).
Fig. 4.9.
Fig. 4.10. Primer extension analysis of *orf2* from fragment 31-2 of *Anabaena* CA indicating potential transcriptional start sites. The same 20-mer was used for both primer extension and for generating the sequence ladder.
Fig. 4.10.
protection/recovery process. Our strategy was to employ subtracted cDNA probes synthesized from RNA extracted from *Anabaena* CA cultures that were exposed to hyperbaric O$_2$ concentration to screen a cosmid genomic library of *Anabaena* CA. Using this approach, we identified a 3.7 kb *HindIII* fragment that hybridized very well with the O$_2$ cDNA probes but hybridized poorly with the N$_2$ cDNA probes from RNA extracted from *Anabaena* CA. Northern hybridizations, using this 3.7 kb fragment to probe RNA prepared from 1% CO$_2$/99% N$_2$ grown cultures and 1% CO$_2$/99% N$_2$ grown cultures followed by 2 h of 1% CO$_2$/99% O$_2$ treatment, confirmed the presence of a 2.2 kb transcript which increased about 5-fold in amount in cells incubated with hyperbaric O$_2$.

Sequence analysis revealed that this fragment contained four ORFs which were transcribed in the same direction. A search of the EMBL and SwissProt protein data bases indicated that the deduced amino acid sequences of these ORFs showed similarity to proteins involved in the synthesis of cell wall polysaccharide in various organisms. ORF 1 shared high sequence identity to the C-terminal halves of RfbP and Ams, the galactosyltransferase protein domain of *Erwinia amylovora*, *Salmonella enterica* and *S. typhimunium*. ORF 1 was found to resemble ExoY of *Rhizobium meliloti* and *Agrobacterium radiobacter* as well as CpsD of *Streptococcus pneumoniae* and Pss4 of *Rhizobium agalactiae* (Table 4.1). These proteins are potential sugar transferases (glycosyltransferases and galactosyltransferases) important for the biosynthesis of polysaccharides in these organisms. Based on the sequence comparison results, ORF 1 from *Anabaena* CA most likely is a sugar transferase that presumably plays a role in polysaccharide synthesis in this organism. The deduced amino acid sequence of ORF 2 of *Anabaena* CA showed greater than 61% identity to RfbD of *Vibrio cholerae* and GDP-D-mannose dehydratase of *E. coli* (YefA) and *Pseudomonas aeruginosa* (*gca* gene product), all of which are involved in the synthesis of nucleotide sugars for O-antigen lipopolysaccharide biosynthesis in these organisms. Therefore, we suggest that ORF 2 is a
putative GDP-D-mannose dehydratase of Anabaena CA that might be a key catalyst involved in the production of active sugars for polysaccharide synthesis. ORF 3 shared sequence similarity to YefB of E. coli, NolK of Azorhizobium caulinodans and ORF 14.8 of Yersinia enterocolitica. NolK also participates in the biosynthesis of nucleotide sugars for polysaccharide production in A. caulinodans. Finally, the search of the protein database revealed that the deduced amino acid sequence of partial ORF 4 shared sequence similarity with the first mannosyl transferase of Salmonella enterica.

In summary, our sequence comparison results indicate that the 3.7 kb fragment from Anabaena CA identified from subtractive hybridization contains genes that encode enzymes for the synthesis of polysaccharides, including sugar transferases and proteins involved in the synthesis of nucleotide sugars.

The 3.7 kb fragment identified from Anabaena CA apparently is very similar to a gene locus identified in previous studies (58). Using transposon mutagenesis, Wolk and colleagues isolated a Fox" (oxygen sensitive nitrogenase) mutant strain B14 of Anabaena 7120 that was also resistant to cyanophages (26, 58). Further studies of this mutant indicated that the transposon 1087b interrupted an ORF whose deduced amino acid sequences was very similar to Salmonella RfbP and Rhizobium ExoY. These authors also indicated that 3' and 5' from the Tn5 marked ORF (58), there were other ORFs which shared sequence similarity with enzymes of polysaccharide biosynthesis. Their results suggest that the lesion in B14 affects polysaccharide synthesis, which appears to be essential for both phage sensitivity and aerobic nitrogen fixation in Anabaena 7120 (58). Thus, these results plus our studies with Anabaena CA, using a substantially different approach, indicate the importance of genes specifying enzymes of polysaccharide synthesis for nitrogenase function in the presence of oxygen.

Northern blot analyses using an Anabaena CA ORF 1 specific probe detected no transcripts in Anabaena CA, suggesting either an inordinately low level of ORF 1 gene
expression or that the ORF 1 transcript is unusually unstable. A long intergenic region, 573 bp, between ORF 1 and ORF 2, and a stem loop structure located downstream of ORF 1, suggests that it is highly unlikely that these two genes are cotranscribed. However, both ORF 2 and ORF 3 hybridized to the same 2.2 kb transcript, indicating that ORF 2 and ORF 3 are cotranscribed. Primer extension experiments detected four putative transcriptional start sites at positions -3, -47, -123, and -157, with respect to the translation start of ORF 2 (Figs. 4.4, 4.10). Since a potential ribosome-binding site, 5'-AAAGGA-3', precedes the ORF 2 translational start by 11 bp and the putative -10 and -35 regions, 5'-TATATT-3' and 5'-TTGACT-3', precedes the ORF 2 translation start by 130 and 146 bp, it is likely that the -123 site is the major transcription start site of ORF 2.

Previous studies established that the metabolism of nitrate repressed the synthesis of heterocysts in Anabaena CA (11). To investigate whether the 2.2 kb mRNA specific to ORF 2 and ORF 3 is enriched in heterocysts, an ORF 2-specific probe was used to analyze transcripts produced by Anabaena CA grown in media containing 10 mM NaNO3. Northern analysis results revealed that the 2.2 kb mRNA was present in undifferentiated (vegetative) cells of Anabaena CA grown under an atmosphere of 1% CO2/99% N2 with or without further O2 treatment (Fig. 4.8). However, the level of this transcript in the vegetative cells was significantly decreased after 2 h of O2 treatment. Comparing the pattern of transcription of cultures that contained only vegetative cells (grown in the presence of nitrate) to cultures which contained both vegetative cells and heterocysts (grown in the presence of N2), it was apparent that induced expression of the ORF 2 and ORF 3 genes in the presence of hyperbaric O2 coincided with the appearance of heterocysts in cultures of Anabaena CA. When 10 mM NH4Cl was added to the medium prior to O2 treatment, the level of this 2.2 kb transcript in Anabaena CA was maintained but did not increase. This is consistent with the fact that ammonia does not repress heterocyst development in strain CA (11), yet its metabolism does block the synthesis of proteins
required for the recovery from \(O_2\)-mediated inactivation (116). The presence of the 2.2 kb transcript in vegetative cells of strain CA suggests that the ORF 2 and ORF 3 genes are involved in cell wall synthesis of vegetative cells, presumably for normal cell growth.

Studies of Wolk and coworkers showed that the diffusion barrier that presumably partially limits the transport of \(O_2\) into heterocysts is composed of an inner glycolipid layer and an outer polysaccharide layer of the heterocyst envelope (86). This important structural feature of heterocysts is thought to provide at least partial protection against \(O_2\)-inactivation of nitrogenase in heterocystous cyanobacteria. \textit{Anabaena} 7120 mutants with aberrant heterocyst envelopes expressed active nitrogenase only under anaerobic conditions (86). In \textit{Anabaena} spp., the heterocyst envelope is poorly developed under low \(pO_2\) and anaerobic conditions (101). Recent studies in \textit{Anabaena flos-aquae} showed that the thickness of the polysaccharide layer and the glycolipid layer of heterocysts is increased with ambient \(pO_2\), suggesting that \(O_2\)-protection of nitrogenase activity requires a thicker envelope (57). It therefore seems reasonable to suggest that the ORFs identified from the subtractive hybridization approach of this investigation may be involved in \(O_2\)-induced thickening of the heterocyst envelope. Consistent with this idea are the findings that these ORFs show similarity to other bacterial cell wall polysaccharide synthesis genes and the fact that ORF 2 and ORF 3 show increased transcription levels under hyperbaric \(O_2\) treatments. Although the transcripts do not appear to be heterocyst-specific, the ORF 2 and ORF 3 genes encoding proteins still might play a role in protecting nitrogenase from \(O_2\)-mediated inactivation, probably by facilitating an increase in the synthesis of polysaccharides that act as an \(O_2\) diffusion barrier in heterocysts.
ACKNOWLEDGMENTS

We thank S. S. Golden for kindly providing us with plasmid pAN4 containing the rRNA genes of *Synechococcus* PCC 6301. We also thank T. Lee for providing us with plasmid pKR616s containing the *R. spheroides* 16 S rRNA gene.
CHAPTER V

Isolation of Up-regulated Genes in the Presence of Hyperbaric Levels of Oxygen in the Cyanobacterium *Anabaena* sp. Strain CA by Subtractive Hybridization

INTRODUCTION

Cyanobacteria, a unique group of prokaryotes, perform oxygenic photosynthesis. When grown in media without fixed nitrogen, some cyanobacteria are capable of aerobic nitrogen fixation. Since all nitrogenase enzymes are oxygen labile, filamentous cyanobacteria like *Anabaena* spp. prevent nitrogenase from O$_2$-mediated inactivation by separating oxygen-evolving photosynthesis and N$_2$ fixation in two distinct cell types, vegetative cells and special differentiated cells called heterocysts (136). Photosynthesis takes place in vegetative cells while N$_2$ fixation is catalyzed by heterocysts under aerobic growth conditions. Thus, mature heterocysts contain nitrogenase but not photosystem II; they also possess a thick cell wall that consists mainly of glycolipids and polysaccharides which are thought to provide a diffusion barrier for oxygen (86). However, despite the protection offered by heterocysts, strong inactivation of nitrogenase occurred in various *Anabaena* spp. under high O$_2$ conditions (93). In *Anabaena* sp. strain CA, previous studies indicated that nitrogenase activity recovered by a process that required protein synthesis, after inactivation in the presence of 1% CO$_2$/99% O$_2$ (93). The metabolism of ammonia, and not nitrate, was found to be involved in the recovery process (116). It was also found that hyperbaric O$_2$ treatments resulted in a decrease in the mobility of
dinitrogenase reductase, from 36 kD to 38 kD, on SDS polyacrylamide gels. The function of this alteration is unknown, however, it may correlate with the acquired tolerance of nitrogenase to oxygen (115,116,125). One key to understanding the mechanisms by which nitrogenase activity recovers from oxygen inactivation would be to identify genes which may play a role in the recovery process. Since the recovery of nitrogenase activity in strain CA required de novo protein synthesis, we thought that this recovery process might be regulated at the transcriptional level in strain CA. Thus, by isolating and cloning up-regulated genes of strain CA under hyperbaric conditions, it might be possible to identify genes required for either the recovery or the protection of nitrogenase activity in *Anabaena* CA. Subtractive hybridization is a powerful method which allows one to isolate differentially expressed genes from two distinct mRNA populations. This strategy has been successfully applied to identify genes from a variety of prokaryotes, such as sporulation genes of *Bacillus subtilis*, virulence genes of *Mycobacterium tuberculosis*, maltose-regulated genes of *Pyrococcus furiosus*, as well as salt induced genes of the cyanobacterium *Anabaena torulosa*, and a light-repressed transcript from the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7002 (1,75,102,126). In our previous studies, we used a subtracted cDNA probe to screen a genomic library of strain CA for preferentially expressed genes under high O2 conditions. Using this strategy, we have successfully isolated some clones that might containing O2 up-regulated genes. However, the size of many of the clones was found to be quite large and most clones were also found to contain rRNA genes, making it difficult to directly use these clones to identify genes in these clones by either sequence and Northern analysis. Thus, among these clones, only a 3.7 kb HindIII fragment was further characterized; it was found to contain O2 up-regulated genes that might be involved in polysaccharide synthesis and aerobic N2 fixation in strain CA (19,58). In the present study, we used a subtractive-PCR method to enrich genes that were不同ially expressed in *Anabaena* CA under high O2 conditions with the goal of
isolating more O₂ up-regulated genes that might be involved in either the protection or the recovery of nitrogenase activity from O₂-mediated inactivation. A subtractive cDNA library of strain CA was constructed by this strategy that contained 4500 clones. By sequence and Northern analyses of 26 randomly chosen cDNA clones from this library, six clones were found to contain O₂ up-regulated genes which might be possibly involved in the protection or recovery of nitrogenase activity from oxygen in *Anabaena* CA.

**MATERIALS AND METHODS**

**Strain and growth conditions.** *Anabaena* sp. strain CA (ATCC 33047) was grown under photoautotrophic conditions in ASP-2 medium (131) (NaCl, 5 g/l) at 39°C with constant illumination. Cultures were bubbled with 1% CO₂/99% N₂ (115).

**RNA extraction and Northern blot analysis.** Total RNA was extracted from mid-exponential growing cultures of *Anabaena* sp. strain CA grown in 100 ml ASP-2 medium bubbled with 1% CO₂/99% N₂, with or without further bubbling with 1% CO₂/99% O₂ for up to 3 h. RNA was extracted and analyzed as previously described (53). RNA (10 µg of each sample) was fractionated on formaldehyde gels and then transferred onto Gene Screen Plus membranes (DuPont, Boston, MA). Hybridizations were carried out in aqueous solutions containing 10% dextran sulfate, 1% SDS, 1 M NaCl, using protocols recommended by the manufacturer. The size of the mRNAs were estimated using an RNA ladder (BRL, Garthersburg, MD). Northern blots were hybridized with random-primed-labeled probes (30).

**cDNA synthesis.** Total RNA was isolated from *Anabaena* CA cultures grown in 1% CO₂/99% N₂ and cultures of *Anabaena* CA initially grown in 1% CO₂/99% N₂ and then switched to 1% CO₂/99% O₂ for 1 h or 2 h, respectively. RNA from 1% CO₂/99% N₂ grown cultures and the RNA mixture from cultures with 1 h or 2 h O₂ treatments were
both used for cDNA synthesis. First strand cDNA was synthesized using 7.5 μg total RNA of *Anabaena* CA as a template in a 20 μl reaction mixture containing 1000 units of SuperScript II reverse transcriptase (BRL), 2 μg random primer [pd(N6) from Pharmacia (Piscataway, NJ)], 40 units of RNasin (Boehringer, Indianopolis, IN), 10 mM dithiothreitol (DTT), 500 μM of each deoxynucleoside triphosphate (dNTP), 50 mM TrisCl (pH 8.3), 75 mM KCl, 3 mM MgCl2. After 1 h at 37°C, the reactions were stopped by chilling the reaction mixture on ice. Nuclease-free water was added to the 20 μl first strand cDNA mixture, and the mixture was directly used for second strand cDNA synthesis. The second strand cDNA was synthesized by *Escherichia coli* DNA polymerase in a total volume of 150 μl containing 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM MgCl2, 10 mM (NH4)2SO4, 0.15 mM DTT, 250 μM of each dNTP, 1 unit DNA ligase, 40 units DNA polymerase I, 2 units RNase H. The second strand synthesis was carried out at 15°C for 4 h. T4 DNA polymerase (15 units) was added to the reaction mixture, and the mixture incubated at 15°C for 15 min. The double-stranded cDNA products synthesized from total RNA of *Anabaena* CA grown in 1% CO2/99% N2 (N2 cDNA) and the mixture of total RNA from strain CA grown in 1% CO2/99% N2 and then transferred to 1% CO2/99% O2 for 1 h or 2 h (O2 cDNA), respectively, were used for subtractions.

**Subtractive enrichment of differentially expressed genes of *Anabaena* strain CA.** As shown in Fig. 5.1, a subtractive-hybridization-polymerase chain reaction (PCR) strategy was used to enrich for O2 up-regulated genes of *Anabaena* strain CA. Subtractive hybridization and PCR amplification was based on the procedures described by Wang and Brown (135) except a rRNA subtraction step was added previous to the PCR amplification of cDNA (Fig. 5.1). To prepare cDNA fragments for PCR amplification, cDNAs were first digested with either Alul or Alul and Rsal, resulting in blunt ended cDNA fragments. The digested cDNA fragments were then ligated with excess amounts of double-stranded phosphorylated oligodeoxynucleotide linkers containing one blunt end and
one 3' protruding end, with an EcoRI site near the blunt end (25-mer, 5'-TAGTCCGAATTCAAGCAAGAGCACA-3'; 21-mer, 3'-ATCAGGCTTAAGTTCGTTCTC-5'). Thus, all linker ligated cDNAs allowed PCR amplification using the same 21-mer. For enrichment of O2 up-regulated genes, cDNA from *Anabaena CA* grown under 1% CO2/99% N2 conditions and cDNA from *Anabaena CA* initially grown in 1% CO2/99% N2 then switched to 1% CO2/99% O2 for 1 and 2 h were both used as "tracer" and "driver" in the subtractive hybridization-PCR reactions.

Removal of rRNA from both "driver" and "tracer" cDNAs was done in the following manner: Plasmid pAN4, which contains 5S, 16S, and 23S rRNA genes from *Synechococcus* 6301 (129) was biotinylated with photobiotin. The biotinylated pAN4 DNA was mixed with N2 ds cDNA and O2 ds cDNA at the ratio of 20:1, heated at 100°C for 3 min, and then allowed to hybridize at 68°C for 20 h. Streptavidin was then added to the hybridization solutions. Biotin-Streptoavidin complexes were removed by phenol-chloroform extraction. The rRNA subtraction step was repeated once. The ds cDNAs (-rRNA) were amplified by PCR; the resulting N2 (-rRNA) ds cDNA and O2 (-rRNA) ds cDNA were then used for subtractive enrichment of differentially expressed genes of *Anabaena CA*.

The N2 (-rRNA) ds cDNA and O2 (-rRNA) ds cDNA were both used as "driver" and "tracer" during the subtractive enrichment process. Driver cDNAs were first digested with EcoRI and then biotinylated with photobiotin. EcoRI digestion previous to biotinylation prevents the driver cDNAs from being amplified in subsequent steps. The biotinylated driver cDNA was mixed with tracer cDNA at a 20:1 ratio. The hybridizations were carried out at 68°C for 20 h (long hybridizations) and the subtraction was performed as described previously. The resulting tracer cDNAs were mixed with biotinylated driver cDNA (1:20), and a short hybridization was carried out at 68°C for 2 h. After the second subtraction, the subtracted tracer cDNAs were amplified by PCR. The amplified tracer
Fig. 5.1. Strategy for constructing a subtractive cDNA library of *Anabaena* sp. strain CA. Total RNA was prepared from cultures of *Anabaena* CA grown in an atmosphere of 1% CO$_2$/99% N$_2$ and also from cultures of *Anabaena* CA that were initially grown in 1% CO$_2$/99% N$_2$ and then transferred to an atmosphere of 1% CO$_2$/99% O$_2$ for 1 h or 2 h, respectively. Double-stranded cDNA synthesized from 7.5 μg total RNA was first digested with *Alu*I and *Rsa*I and then ligated to *EcoRI* linkers. Biotinylated driver (100 μg) and nonbiotinylated tracer (5 μg) DNAs were mixed and hybridized at 68°C for 20 h (long hybridizations) or 2 h (short hybridizations). Streptavidin (SA) was added to the hybridized cDNA solution to bind the biotinylated DNA, then protein and protein-DNA complexes were removed by phenol-chloroform extraction. The subtracted ds cDNA was PCR amplified, digested with *EcoRI*, and then clone into pBluescript SK+ vector to construct a cDNA library.
Fig. 5.1.
cDNA. O$_2$ (-N$_2$) ds cDNA, was digested with EcoRI, cloned into the pBluescript SK+ vector (Strategene) and was used to transform E. coli JM109 cells to generate a subtractive cDNA library containing O$_2$ up-regulated genes of *Anabaena* CA.

**DNA sequencing.** Plasmids containing cDNA inserts were used for sequencing. The nucleotide sequences of both strands were determined by using T3 and T7 primers, and terminator cycle sequencing kits (Applied Biosystems, Inc., Foster City, CA). Sequence reactions mixture were analyzed with an ABI PRISM 310 Genetic Analyzer. Sequence data were analyzed by using the sequence analysis programs of the University of Wisconsin Genetics Computing Group (GCG).

**RESULTS**

**Enrichment of cDNAs containing O$_2$ up-regulated genes of *Anabaena* sp. strain CA.** To identify more O$_2$ up-regulated genes of *Anabaena* sp. CA that might be involved in the protection and/or recovery of nitrogenase, a subtractive cDNA library was constructed based on the method described by Wang and Brown (135) (Fig. 5.1). The subtracted O$_2$ ds cDNA was cloned into pBluescript SK+ vector to generate a cDNA library containing 4500 clones. DNA prepared from one tenth of the clones of this library was used for Southern hybridizations; a rRNA gene probe (containing 5 S, 16 S, and 23 S rRNA genes of *Synechococcus* 6301), a N$_2$(-O$_2$) cDNA probe, and a O$_2$(-N$_2$) cDNA probe were employed to check the subtraction efficiency. Southern hybridization results revealed that 72% of these clones hybridized poorly to the N$_2$(-O$_2$) cDNA probe, and 43% of them did not hybridize to the rRNA probe. These results suggested that the subtraction of rRNA and N$_2$ cDNA worked but was not complete. Twenty six recombinants which
hybridized significantly more strongly with the O\(_2\) (-N\(_2\)) cDNA probe compared with the N\(_2\) (-O\(_2\)) cDNA probe were randomly picked for sequence and Northern analysis.

**Sequence and Northern analyses of selected cDNA clones of Anabaena sp. strain CA.** To identify genes encoded by these randomly chosen cDNA clones, the sequence of these cDNA clones was determined using T3 and T7 primers. The deduced amino acid sequences of these clones was compared to the known protein data base using the Fasta and Blast programs of the GCG package. Sequence analysis of these selected cDNA clones revealed that half of them contained rRNA sequences, however, the rest of them were unique clones. These results are summarized in Table 5.1. Based on sequence analysis, these clones may be divided into at least four groups, group 1 containing genes involved in nitrogen fixation; group 2, containing genes probably involved in electron transport; group 3, containing a gene probably involved in polysaccharide synthesis; group 4, containing genes involved in photosynthesis. The search of GenBank and EMBL databases found no protein similar to deduced sequences of clone 420, 427, and 344, respectively. Sequence analysis established that clones 328 and 341 were identical. The deduced amino acid sequences of clones 328 and 341 exhibited greater than 90% identity with the \textit{nifH} gene product (dinitrogenase reductase) of various filamentous cyanobacteria (5,33,77,83) (Fig. 5.2). Sequence analyses also established that the 5' portion of the deduced amino acid sequence of clone 8 was similar to previously sequenced ORFs in \textit{nif} gene clusters of 3 cyanobacterial strains, including ORF 3 downstream of \textit{nifK} in \textit{Anabaena} 7120 (Accession No. U47055), ORF 4 located downstream of \textit{nifK} in \textit{Fischerella} sp. (Accession No. U49514) and also an ORF 1 located downstream of \textit{nifK2} of \textit{Anabaena variabilis} (128). The function of the ORFs in these strains is presently unknown. Sequence analyses also indicated that clone 419 was similar to potential FMN-proteins of \textit{Rhodobacter capsulatus}, previously suggested to be involved in electron transport to nitrogenase (108,110). The deduced amino acid sequence of clone
Table 5.1. Characteristics of selected cDNA clones.

<table>
<thead>
<tr>
<th>Clone No.</th>
<th>Presumptive protein*</th>
<th>Transcript size (kb)</th>
<th>Expression in O2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>ORF 3 of <em>Anabaena</em> 7120 &lt;br&gt; ORF 1 of A. variabilis &lt;br&gt; ORF 4 of <em>Fischerella</em> sp.</td>
<td>ND c</td>
<td>8 fold increase</td>
</tr>
<tr>
<td>22</td>
<td>photosystem I reaction center protein (PsaA)</td>
<td>ND</td>
<td>11 fold increase</td>
</tr>
<tr>
<td>349</td>
<td>polysaccharide synthesis related proteins</td>
<td>1.5</td>
<td>induced in heterocysts under 1% CO2/99% O2</td>
</tr>
<tr>
<td>419</td>
<td>FMN containing protein involved in electron transport to nitrogenase</td>
<td>1.1</td>
<td>2 fold increase</td>
</tr>
<tr>
<td>328</td>
<td>dinitrogenase reductase</td>
<td>4.6, 2.5, 0.9</td>
<td>2 fold increase (nifH probe from <em>Anabaena</em> 7120)</td>
</tr>
<tr>
<td>341</td>
<td>dinitrogenase reductase</td>
<td>4.6, 2.5, 0.9</td>
<td>2 fold increase (nifH probe from <em>Anabaena</em> 7120)</td>
</tr>
<tr>
<td>286</td>
<td>D1 protein of photosystem II</td>
<td>1.3</td>
<td>3 fold decrease</td>
</tr>
<tr>
<td>9</td>
<td>CP43 protein (PsbC) of photosystem II</td>
<td>2.9</td>
<td>3 fold decrease</td>
</tr>
<tr>
<td>10</td>
<td>CP43 protein (PsbC) of photosystem II</td>
<td>2.9</td>
<td>3 fold decrease</td>
</tr>
<tr>
<td>122</td>
<td>pheromone binding protein</td>
<td>2.8, 1.9, 1.5</td>
<td>no change</td>
</tr>
<tr>
<td>344</td>
<td>Not identified</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>420</td>
<td>Not identified</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>427</td>
<td>Not identified</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Homologies were determined by using the Blast and Fasta programs of the GCG package.

bThe level of transcription is quantitated using the Storm instrument (Molecular Dynamics Inc., Sunnyvale, CA).

cND, not determined.
Fig. 5.2. Comparison of the deduced amino acid sequence of a portion of clone 328 from *Anabaena* sp. strain CA to the dinitrogenase reductase (*nifH* product) from *Anabaena* sp. strain L31 (L31), *Anabaena* 7120 (7120), *Nostoc* sp. strain PCC 6720 (6720) and *Plectonema boryanum* (Pb). The alignment was determined using the Pileup program of the GCG package. Residues identical to all four proteins are shaded.
349 showed similarity to proteins involved in polysaccharide biosynthesis in different organisms, such as the capsular polysaccharide synthesis related protein of *Klebsiella pneumoniae*, mannosyl transferase B of *E. coli*, and the lipopolysaccharide biosynthesis related protein in *Haemophilus influenzae* (Accession No. U36398) and in *Salmonella typhimurium* (2,71,123). Besides proteins involved in nitrogen fixation and in polysaccharide synthesis, sequence analysis also found four cDNA clones from *Anabaena CA* containing genes which encode proteins of the photosystems. The deduced amino acid sequence of clone 22 shared about 90% identity with the PsaA protein of *Anabaena variabilis*, and with PsaA of *Synechococcus* spp. These are reaction-center proteins of photosystem I that mediate light-induced electron transfer from plastocyanin or cytochrome c553 to ferredoxin. Fig. 5.3 shows the alignment of clone 22 with the *psaA* gene product from *Anabaena variabilis* and various unicellular strains (17,87,112,113). Clone 286 was found to contain *psbA* genes which code for the D1 protein of photosystem II. Fig. 5.4 presents the alignment of the deduced amino acid sequence of clone 286 from *Anabaena CA* with PsbA from unicellular and filamentous strains (82,132). Clones 9 and 10 were found to be highly homologous to the *psbC* product (CP43), a chlorophyll-binding antenna protein of the photosystem II reaction. The alignment of clone 9 with the CP43 protein from *Anabaena 7120* and with CP 43 proteins from *Synechococcus* spp. is shown in Fig. 5.5 (24,39,65). Sequence analysis also indicated that clone 122 shared similarity to proteins belonging to the bacterial extracellular solute-binding protein family, such as the XP55 protein from *Streptomyces lividans* and a probable pheromone binding protein from the conjugative plasmid of *Enterococcus faecalis* (15,105).

To determine whether the expression of genes within these clones was induced in *Anabaena CA* in the presence of hyperbaric O2, *EcoRI* inserts were used to probe Northern blots of RNA prepared from cultures of *Anabaena* sp. strain CA grown in 1% CO2/99% N2 or in cultures initially grown in 1% CO2/99% N2 and then transferred to 1% CO2/99%
Fig. 5.3. Comparison of the deduced amino acid sequence of part of clone 22 from *Anabaena* CA to the PsaA proteins from *Anabaena variabilis* (Av), *Synechococcus vulcanus* (Sv), *Synechococcus elongatus naegeli* (Se), *Synechocystis* 6803 (6803), and *Synechococcus* PCC 7002 (7002). The alignment was determined using the Pileup program of the GCG package. Identical residues are shaded.
Fig. 5.4. Comparison of the deduced amino acid sequence of clone 286 from *Anabaena* CA to PsbA from *Synechococcus elongatus* naegeli (Se), *Fremyella diplosiphon* (Fd), and *Anabaena* 7120 (7120). The alignment was determined using the Pileup program of the GCG package. Identical residues are shaded.
<table>
<thead>
<tr>
<th>Clone</th>
<th>Sequence</th>
<th>Anabaena sp. strain 10</th>
<th>Anabaena 7120</th>
<th>Anabaena 6803</th>
<th>Anabaena 7942</th>
<th>Synechocystis 6803</th>
<th>Synechococcus 7942</th>
</tr>
</thead>
<tbody>
<tr>
<td>#10</td>
<td></td>
<td></td>
<td>FGGETMRFWD FQGPWLEPLR GPNGLDLDKI KNDIQPWQAR RAAEYMTTHAP</td>
<td>FGGETMRFWD FQGPWLEPLR GPNGLDLDKI KNDIQPWQAR RAAEYMTTHAP</td>
<td>FGGETMRFWD FQGPWLEPLR GPNGLDLDKI KNDIQPWQAR RAAEYMTTHAP</td>
<td>FGGETMRFWD FQGPWLEPLR GPNGLDLDKI KNDIQPWQAR RAAEYMTTHAP</td>
<td>FGGETMRFWD FQGPWLEPLR GPNGLDLDKI KNDIQPWQAR RAAEYMTTHAP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FGGETMRFWD FQGPWLEPLR GPNGLDLDKI KNDIQPWQAR RAAEYMTTHAP</td>
<td>FGGETMRFWD FQGPWLEPLR GPNGLDLDKI KNDIQPWQAR RAAEYMTTHAP</td>
<td>FGGETMRFWD FQGPWLEPLR GPNGLDLDKI KNDIQPWQAR RAAEYMTTHAP</td>
<td>FGGETMRFWD FQGPWLEPLR GPNGLDLDKI KNDIQPWQAR RAAEYMTTHAP</td>
<td>FGGETMRFWD FQGPWLEPLR GPNGLDLDKI KNDIQPWQAR RAAEYMTTHAP</td>
</tr>
</tbody>
</table>

Fig. 5.5. Deduced amino acid sequence of a portion of clone 10 from Anabaena sp. strain CA compared with the CP43 protein (psbC gene product) of Anabaena 7120 (7120), Synechocystis 6803 (6803), and Synechococcus 7942 (7942). The alignment was determined using the Pileup program of the GCG package. Identical residues are shaded.
O₂ for 2 h. These results are summarized (Table 5.1). The Northern blot experiments confirmed that six clones contained genes whose expression was significantly induced in *Anabaena* CA after O₂ treatment, including clones 8, 22, 349, 419, 328, 341. In particular, RNA blots probed with *EcoRI* fragments from clones 8, 22, and 419 yielded about 8-fold (data not shown), 11-fold, and 2-fold increases, respectively, in the level of transcription after 2 h in the presence of hyperbaric O₂ (Fig. 5.6). When probed with an *EcoRI* fragment from clone 349, a significant increase in the level of transcription was observed in strain CA after 2 h in hyperbaric O₂ (Fig. 5.7). The accumulation of clone 349 specific transcript in whole filaments containing both heterocysts and vegetative cells under high hyperbaric O₂ conditions presumably was due to the induced transcription of clone 349 specific genes in heterocysts of strain CA, since the level of its transcript did not change in filaments containing only vegetative cells when the organism was grown in the presence of nitrate under 1% CO₂/99% N₂ with or without a further 2 h O₂ treatment. The deduced amino acid sequence of clone 328 (identical to clone 341) showed a high level of identity to the *nifH* product of various filamentous strains, including *Anabaena* 7120 (Fig. 2). For our Northern analysis, instead of using sequences from clone 328 and 341, we used a *nifH* probe from *Anabaena* 7120 (98). When RNA blots were probed with *nifH* from *Anabaena* 7120, there was about a 2-fold increase in the level of transcription in *Anabaena* CA after 2 h O₂ of treatment (Fig. 5.8). In addition, all of the transcript obtained from cells grown in 1% CO₂/99% N₂ was in the lower molecular weight (0.9 kb *nifH* transcript) form while there were significant amounts of higher molecular weight transcripts (*nifHDK* transcripts) in cells switched to the hyperbaric O₂ atmosphere. It seems that the higher molecular weight transcripts were degraded in cells grown under 1% CO₂/99% N₂ conditions. The reason for this degradation is not clear. Northern analyses also indicated that sequences of clones 9, 10, 286, and 122 were not induced in *Anabaena* CA. Instead of accumulation, the transcriptional level decreased about 3-fold in the hyperbaric O₂
Fig. 5.6. Northern analysis using clones 22 and 419 from *Anabaena* sp. strain CA. Lanes labeled N2 or O2 contained, respectively, 10 μg total RNA from cells grown in 1% CO2/99% N2 or in 1% CO2/99% N2 and then transferred to 1% CO2/99% O2 for 2 h. The EcoRI inserts from clones 22 (A), and 419 (B) were used to probe the RNA blots. The level of transcription was quantitated using a Storm 840 imaging analysis instrument (Molecular Dynamics, Inc., Sunnyvale, CA).
Fig. 5.6.
Fig. 5.7. Northern blot analysis of whole filaments containing both vegetative cells and heterocysts (A) and filaments containing only vegetative cells (B) of Anabaena sp. strain CA using an EcoRI fragment of clone 349. Lane labeled N2 or O2 contained, respectively, 10 μg total RNA prepared from cells grown in 1% CO2/99% N2 or 1% CO2/99% N2 and then transferred to 1% CO2/99% O2. Total RNA was prepared from cultures grown in ASP-2 medium in the absence of fixed nitrogen (A) or supplemented with 10 mM NaNO3 to yield filaments that contained only vegetative cells (B).
Fig. 5.7.
Fig. 5.8. Northern blot analysis of *nifH* in *Anabaena* CA. Lanes labeled N₂ or O₂ contained, respectively, 10 μg total RNA from cells grown in 1% CO₂/99% N₂ and 1% CO₂/99% N₂ and then switched to 1% CO₂/99% O₂ for 2 h. A *nifH* probe from *Anabaena* 7120 (pAn154.3) was used for the hybridizations. The level of *nifH* transcription in *Anabaena* CA was quantitated using a Storm 840 imaging analysis instrument (Molecular Dynamics, Inc., Sunnyvale, CA).
atmosphere, while transcripts specific to sequences of clone 122 did not change during O$_2$ treatment (results not shown). Therefore, these clones are not up-regulated in the presence of O$_2$.

**DISCUSSION**

The ability of nitrogenase to recover from O$_2$-mediated inactivation was found in several *Anabaena* spp. including *Anabaena* sp. strain CA (93). In strain CA, Pienkos et al. observed that recovery after exposure to hyperbaric O$_2$ concentrations was not entirely dependent on *de novo* synthesis of nitrogenase (93). To elucidate the molecular basis for the regulation of the recovery process, it will be certainly necessary to identify genes which play a role in the recovery process. A previous screening of a genomic library of *Anabaena* CA, using subtracted cDNA probes, resulted in the identification of cosmid clones of strain CA that might contain O$_2$ up-regulated genes. However, these clones also contained rRNA genes and the size of their inserts ranged from 2-6 kb, making it difficult for direct sequencing and Northern analysis to identify genes on these fragments. Among these clones, only a 3.7 kb fragment subcloned from one clone was further analyzed. This fragment was found to contain O$_2$ up-regulated genes that were probably involved in polysaccharide synthesis and aerobic N$_2$ fixation in strain CA (19,58).

In the present study, to identify more O$_2$ up-regulated genes of *Anabaena* CA that might be involved in either protection or recovery of nitrogenase activity in *Anabaena* spp., a subtractive cDNA library was constructed based on the methods described by Wang and Brown (135). Twenty six clones were randomly chosen for sequence and Northern analysis which hybridized significantly better with the O$_2$(-N$_2$)cDNA probe than with the N$_2$(-O$_2$)cDNA probe. Sequence analysis indicated that half of these clones contained 16 S or 23 S rRNA genes; the rest of them were unique clones. RNA blot analysis of nine
clones established that six clones, 8, 22, 349, 328, 341, and 419, all contained O₂ up-regulated genes. Three clones (9, 10, and 286) were found to exhibit decreased transcription in the presence of hyperbaric O₂. This may be due to our subtraction procedures; thus, instead of using multiple cycles of long hybridization to remove rare sequences and short hybridization to remove abundant sequences (described by Wang and Brown), we only used one long hybridization and one short hybridization to remove sequences present in both N₂ cDNA and O₂ cDNA. Apparently, this is not enough to remove all the common sequences shared by N₂ cDNA from O₂ cDNA.

Sequence analysis indicated that clones 9 and 10 contained the psbC sequence and clone 286 contained a psbA sequence. psbA codes for photosystem II reaction center protein D1 while psbC codes for CP43, an antenna protein of photosystem II. The expression of these genes showed about a 3-fold decrease in strain CA. Since illuminated cultures of strain CA were incubated under hyperbaric O₂ conditions, it might be expected that proteins encoded by psbA and psbC might have high turnover rates due to the increase of reactive O₂ species and the potential subsequent damage to the photosystem. However, the expression of six O₂ up-regulated clones in strain CA was found to show a 2- to 11-fold increase in the presence of hyperbaric O₂ (Table 1). Sequence analysis revealed that the six O₂ up-regulated clones were nifH (clones 328 and 341), nif related (clone 8), psaA (clone 22), genes that might be involved in polysaccharide synthesis (clone 349) and a gene involved in electron transport to nitrogenase (clone 419). The potential role of these genes and their products in the protection and recovery of nitrogenase activity in Anabaena is unknown. However, it is certainly reasonable to assume that recovery from O₂-mediated inactivation might require these and other genes that are important for nitrogenase function, as well as genes that encode proteins that contribute to the unique environment found in the heterocysts. Similar to fragment 31-2 identified from previous studies (Chapter IV), the accumulation of clone 349 specific mRNA appears to be due to increased expression in
heterocysts in the presence of hyperbaric O2. The deduced sequences of clone 349 resembled the ORF 7 located in *cps* region of *Klebsiella pneumoniae*, mannosyl transferase B of *E. coli*, and lipopolysaccharide 1,2-N-acetylglucosaminetransferase of *Salmonella typhimurium*, which have been implicated in polysaccharide biosynthesis in these organisms. Thus, the presence of clone 349 in our subtractive cDNA library suggests that it might play some role in the thickening of heterocyst cell walls, which contribute towards maintaining an anaerobic environment for N2 fixation (19,58). The presence of clones 8, 341, 328, and 419 in our subtractive library suggests that products encoded by genes of these clones might possibly be involved in electron transport to nitrogenase under hyperbaric O2 conditions. Alternatively, perhaps the products of these genes might participate in electron transport systems for either respiration or photosynthesis or in maintaining the N2-fixing capacity by new synthesis of enzymes, including nitrogenase. We can not exclude the possibility that the *nifH* gene that we cloned is an alternative nitrogenase, since the *nifH* gene of both dinitrogenase reductase and the alternative dinitrogenase reductase from various organisms was found to be highly conserved (7).

The presence of the *psaA* gene in our subtractive library suggests that photosystem I could also possibly be involved in the protection of nitrogenase in *Anabaena* CA, probably by providing ATP that is necessary for N2 fixation and other biosynthetic processes such as polysaccharide synthesis, which in turn might contribute to the putative O2 diffusion barrier of heterocysts or in decreasing O2 concentrations in heterocysts since PSI is also closely linked to the respiratory electron transport system (28).

In summary, to identify genes involved in either protection or recovery of nitrogenase activity, we have used a subtraction-PCR method to enrich for sequences that were differentially expressed in *Anabaena* sp. strain CA under hyperbaric O2 conditions. After screening 26 clones from the subtracted cDNA library, we found six of these to contain genes that were up-regulated in the presence of hyperbaric O2. These genes might
be involved in polysaccharide synthesis, electron transport, photosystem synthesis, and nitrogen fixation. Thus, this subtractive cDNA library strategy has proven to be effective for cloning differentially expressed genes from *Anabaena* sp. strain CA. The precise functions of these genes identified in this study and their role in protection or in recovery of nitrogenase activity in *Anabaena* sp. strain CA awaits further investigation.
CHAPTER VI

CONCLUDING REMARKS

The ability to fix or reduce atmospheric dinitrogen to ammonia is limited to a diverse group of organisms, all of which are prokaryotes. Nitrogen fixation is catalyzed by an enzyme complex, nitrogenase, which is composed of two component proteins, the Mo-Fe protein (dinitrogenase) and the Fe protein (dinitrogenase reductase). The Mo-Fe protein and Fe-protein are both damaged by exposure to \( \text{O}_2 \) (16,80,103). Cyanobacteria are phototrophic, gram-negative bacteria which possess both photosystem I and II, hence, like plants, they are able to photolyse water to generate \( \text{O}_2 \) (50). It has been known for over a century that some cyanobacteria can also fix \( \text{N}_2 \) under vigorously aerobic conditions. To prevent nitrogenase from \( \text{O}_2 \)-mediated damage, these organisms have evolved diverse strategies to cope with oxygen. For unicellular and non-heterocystous filamentous strains, temporal separation of \( \text{N}_2 \) fixation from photosynthesis, \( \text{O}_2 \) consumption and respiration has been noted (28,36,44,79,121). In heterocystous filamentous strains such as Anabaena spp., the oxygen-sensitive nitrogenase is confined in the differentiated cells, heterocysts, while oxygeonic photosynthesis occurs only in vegetative cells (136). Thus, mature heterocysts contain active nitrogenase but lack photosystem II. These differentiated cells also possess a thick envelope consisting of glycolipids and polysaccharides, which is thought to provide at least a partial diffusion barrier for \( \text{O}_2 \) (86).

Some time ago it was shown that intracellular nitrogenase activity from a variety of Anabaena spp. recovers from oxygen-mediated inactivation within a short time after an initial inactivation by hyperbaric \( \text{O}_2 \) (93). For Anabaena sp. strain CA, hyperbaric \( \text{O}_2 \)
incubation also resulted in a mobility shift of dinitrogenase reductase on SDS polyacrylamide gels, from a position characteristic of a 36 kD protein to one of 38 kD. This alteration appeared to relate to the presence of oxygen in such cultures and previous results suggested that this alteration might also render nitrogenase more O2 tolerant. The recovery of nitrogenase activity in strain CA in the presence of hyperbaric oxygen was found to require active protein synthesis, but was not completely dependent on de novo synthesis of nitrogenase proteins, suggesting that the synthesis of some other proteins might be important in recovery (93,115,125). The goal of this dissertation was to gain a further understanding of the intracellular components and strategies two diverse strains employ to cope with the oxygen problem, the unicellular organism *Synechococcus* sp. RF-1 and the filamentous heterocystous cyanobacterium, *Anabaena* sp. strain CA. Strain RF-1 exhibits a circadian rhythm of N2 fixation when grown under diurnal light-dark cycles (43,53,54,), *Anabaena* sp. strain CA does not. This organism, however, has the inherent ability to facilitate nitrogenase recovery from an initial inactivation by O2.

The pattern of *nifH* and *rbcL* transcription and the pattern of Fe protein modification in strain RF-1 were examined during 12 h light/12 h dark cycles as well as under continuous light conditions. When grown under continuous light conditions, *nifH* transcripts and *rbcL* transcripts in strain RF-1 were continuously detected throughout the 24 h experimental period. However, when grown under 12 h light/12 h dark conditions, consistent with previous results, *nifH* transcripts were present mainly in the dark. The *rbcL* transcripts were detected in both the light and the dark, but with significantly greater expression during the light cycles. Temporal separation of N2 fixation in the dark and photosynthesis in the light has been suggested as a potential strategy for *Synechococcus* spp. and other unicellular strains to protect their nitrogenase from the damaging effects of photosynthetically generated O2 (28). Since RubisCO is one of the key enzymes of the reductive pentose phosphate pathway for CO2 assimilation in cyanobacteria, the significant
accumulation of the rbcL mRNA in the light and the presence of nifH mRNA mainly during the dark phase provides some evidence for the temporal separation hypothesis (Chapter II).

To determine if posttranslational modification might also regulate the nitrogenase activity in the dark and the light, antiserum against the *R. rubrum* Fe protein was used to probe the Fe protein of nitrogenase of strain RF-l during the light-dark cycle. The appearance of the Fe protein during the dark phase correlated with nitrogenase activity. In cells collected during the dark phase, two bands (37 and 39 kD) cross-reacted with the Fe protein antiserum. Upon illumination, there was a conversion of all the Fe protein to the modified form, or 39 kD form, which rapidly disappeared from cell extracts during the light period. These results indicate that nitrogenase activity, present during the dark cycle, results from de novo synthesis of nitrogenase; the rapid degradation of the Fe protein upon entering the light phase may result from activating or inducing a protease which appears to be specific to the modified 39 kD Fe protein (Chapter II).

To get a better understanding the mechanisms by which *Anabaena* CA protects its nitrogenase from the deleterious effects of O2 and mediates the recovery of its nitrogenase activity. Our initial attempt was to identify potential protein components and genes that might be important for the protection or recovery of nitrogenase activity from O2-mediated inactivation in strain CA, using both the wild-type and O2 sensitive mutants of strain CA. Potential protein components were sought by comparing two dimensional gel protein profiles of these mutants with wild-type strain CA, the eventual goal being to identify requisite structural genes by complementing O2-sensitive mutants of strain CA with a genomic library of wild-type strain CA. Nine O2 sensitive mutants of wild-type strain CA were isolated. All these mutants were able to grow microaerobically under nitrogen-fixing conditions. These mutants showed different levels of O2 sensitivity, suggesting that the mutants might have lost the ability to either protect nitrogenase from O2-mediated inactivation or to recover from O2-mediated inactivation (Chapter III). However, our
attempts to set up a gene transfer system for *Anabaena* sp. strain CA was not successful. Thus, identifying the genes that might be involved in the protection and/or recovery process using the approach of complementing O2 sensitive mutants was not feasible.

In order to identify potential protein components that might be involved in the protection/recovery of nitrogenase, 2 D gel electrophoresis was performed to compare the total protein profiles of wild-type strain CA and mutant strain N102, a strain that had lost its ability to protect/recover its nitrogenase from O2-mediated inactivation. Fifteen proteins were identified by this strategy. In both the wild-type and strain N102, significant changes in the appearance and disappearance of these proteins was observed during the time cells were exposed to hyperbaric O2. The 36 kD (C2) and the 38 kD (C3) Fe protein of nitrogenase in the wild type and in strain N102 were identified. In wild-type CA, both the 36 kD and 38 kD proteins increased in amount after whole filaments were exposed to hyperbaric O2, suggesting that nitrogenase synthesis in strain CA was not repressed by exposure to O2. Also in wild-type CA, the addition of chloramphenical during O2 treatment resulted in a significant increase in the amount of the Fe protein, however in its 38 kD (presumably inactivated) form. These results support previous findings which indicated recovery of activity is not completely dependent on *de novo* synthesis of nitrogenase. Other newly synthesized proteins, involved either in the activation of the 38 kD Fe protein or in the protection of the 36 kD protein from inactivation, might be important for this recovery process. A significant decrease in both the 36 kD and 38 kD Fe protein of nitrogenase in strain N102 indicated that the inability of this mutant to recover its nitrogenase activity after O2 treatment may be due to rapid degradation or some failure of *de novo* synthesis of the 36 kD protein. Also a C5 protein, identified as 50 S ribosomal protein L21 by N-terminal amino acid sequence analysis, was present in much smaller amounts in strain N102, suggesting that one reason that nitrogenase in this mutant could not recover from O2-mediated inactivation might be related to some general inability of this

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mutant to synthesize protein during hyperbaric oxygen stress (Chapter III). The proteins identified from 2D gels are potential candidates involved in the protection and/or recovery of nitrogenase activity; they may somehow be involved specifically with this function. If they are not directly involved, their study might still provide useful information relative to O\textsubscript{2} stress in these organisms. It should be pointed out that \textit{Anabaena} spp. and other heterocystous forms often face supersaturated oxygen concentrations in the natural environment. Unfortunately, the proteins in question were not present in great abundance, making it difficult to isolate and identify the genes which encode them.

A genetic screen is the traditional method employed to identify genes involved in complicated biological processes. In particular, subtractive hybridization methods have been effectively applied to identify differentially expressed genes from a variety of microorganisms (1,75,102,126). Since the recovery of nitrogenase activity in strain CA required \textit{de novo} protein synthesis (93), this recovery process might also be regulated at the transcriptional level. Thus, by isolating up-regulated genes of strain CA under hyperbaric conditions, it might be possible to identify genes required for either the recovery or the protection of nitrogenase in \textit{Anabaena} CA. Two subtractive hybridization strategies have been used to clone differentially expressed genes of \textit{Anabaena} CA in the presence of hyperbaric O\textsubscript{2}. The first strategy involved screening the genomic library of \textit{Anabaena} CA with a subtracted cDNA probe. The second strategy involved constructing a subtractive cDNA library of \textit{Anabaena} CA. A 3.7 kb \textit{HindIII} fragment of strain CA containing up-regulated genes under high O\textsubscript{2} concentrations was identified by screening the genomic library of strain CA with a subtracted cDNA probe. Northern hybridizations, using this 3.7 kb fragment probe, confirmed that the level of a 2.2 kb mRNA was increased in strain CA in response to hyperbaric O\textsubscript{2} (Chapter IV). Sequence analysis revealed four open reading frames located in this 3.7 kb fragment. These proteins are similar to sugar transferases and other proteins used by bacteria for the synthesis of nucleotide sugars that
are involved in polysaccharide synthesis. The predicted amino acid sequence of ORF 1 shared identity with sugar transferases of various organisms including the RfbP, ExoY, CpsD, and Pss4 proteins. Upstream of ORF 1, a partial ORF, ORF 4, was also found and shown to be similar to the first mannosyl transferase of *Salmonella enterica*. The deduced amino acid sequences of ORF 2 exhibited about 61% identity to RfbD of *Vibrio cholerae* and GDP-D-mannose dehydratases of *E. coli* (YefA) and *Pseudomonas aeruginosa* (GCA). ORF 3 shared significant sequence identity with NolK of *Azorhizobium caulinodans*, ORF 14.8 of *Yersinia enterocolitica*, and YefB of *E. coli*, all of which are also involved in the biosynthesis of polysaccharides (Chapter IV).

Northern hybridization experiments using probes specific to these ORFs revealed that the 2.2 kb transcript that accumulated in cells of *Anabaena* CA in response to hyperbaric O₂ atmospheres contained both ORF 2 and ORF 3 mRNA. Further Northern analysis found the level of this 2.2 kb transcript declined in vegetative cells of *Anabaena* CA in response to hyperbaric O₂ treatment, suggesting that the accumulation of this 2.2 kb transcript in differentiating cultures of strain CA might be due to increased expression of ORF 2 and ORF 3-encoding genes in the heterocysts. The precise function of these ORFs in *Anabaena* CA is not known at present. However, the results of sequence and Northern blot analyses of these ORFs suggest that the ORFs may be implicated in some way in protecting nitrogenase from O₂-mediated inactivation in strain CA, probably by facilitating the synthesis of thickened cell walls of heterocysts, thereby enhancing the ability of this structure to act as an O₂-diffusion barrier (Chapter IV).

To isolate additional genes that might be important for the recovery and/or protection of nitrogenase activity from O₂-mediated inactivation, a subtractive-PCR method was also used to enrich for genes that would be differentially expressed in *Anabaena* CA under hyperbaric O₂ conditions. A subtractive cDNA library was constructed by this strategy that contained 4500 clones. Among these, twenty six clones were chosen for
further sequence and Northern analysis since they all hybridized significantly more strongly with the O2(-N2) cDNA probe compared with the N2(-O2) cDNA probe. Sequence analysis of the 26 selected cDNA clones revealed that half of them contained 16 S and 23 S rRNA genes, while the rest of them were unique clones. Three clones, containing psbA and psbC genes, respectively, were actually found to exhibit decreased transcription under high O2 concentrations. The reason these clones were selected may be due to incomplete subtraction. However, Northern analysis confirmed that six clones contained genes that were up-regulated in the presence of hyperbaric O2. Sequence analysis revealed that the six O2 up-regulated clones were nifH (clones 328, 341), nif related (clone 8), psaA (clone 22), a gene that might be involved in polysaccharide synthesis (clone 349), and a gene involved in electron transport to nitrogenase (clone 419). The potential role of these genes and their products in the protection and recovery of nitrogenase activity is not known but it is reasonable to assume that recovery from O2-mediated inactivation might require genes involved in the function of nitrogenase as well as genes that contribute to the unique environment found in heterocysts. The accumulation of clone 349 specific mRNA seems to be due to increased expression in heterocysts in the presence of hyperbaric O2. Thus, the presence of clone 349 in our subtractive cDNA library suggests that it could have some function in thickening the cell walls of heterocysts, which might in turn serve as a diffusion barrier for O2. The presence of clones 8, 341, 328, and 419 in our subtractive library suggests that the products of these genes might participate in electron transport systems or in maintaining the N2-fixing capacity by new synthesis of enzymes, including nitrogenase, under hyperbaric O2 conditions. However, at this point we cannot exclude the possibility that the nifH gene that we cloned from our subtractive library might encode an alternative nitrogenase, since genes specific to both dinitrogenase reductase and alternative dinitrogenase reductase from various organisms have been found to be highly conserved (7). The presence of psaA in our subtractive cDNA library suggests that photosystem I
could possibly be involved in the protection of nitrogenase in *Anabaena* CA, probably by providing ATP required for N\(_2\) fixation and other biosynthetic processes including polysaccharide synthesis, which might in turn contribute to the diffusion barrier of heterocysts. Additional ATP may also be required for some O\(_2\) scavenging function in heterocysts since PSI is also linked to the respiratory electron transport system (Chapter V).

The results obtained from the studies of *Anabaena* CA mutants and 2 D gel comparisons support the previous studies of strain CA by Peinkos et al. (93), that is, *de novo* synthesis of nitrogenase is not sufficient to maintain the N\(_2\)-fixing capacity of *Anabaena* CA under hyperbaric O\(_2\) conditions. Thus, some other newly synthesized proteins are probably involved in the functioning of nitrogenase under hyperbaric O\(_2\) atmosphere and might be important for this recovery process. Using the subtractive hybridization strategy, we identified several up-regulated genes of *Anabaena* CA incubated under hyperbaric O\(_2\) conditions, including genes related to heterocyst polysaccharide synthesis, *nifH*, and *nif* related gene, genes involved in electron transport and photosystem I. For *Anabaena* CA, an organism that have well developed heterocysts, to maintain its N\(_2\)-fixing capacity under hyperbaric O\(_2\) atmosphere a thicker heterocyst cell wall might help to protect its nitrogenase from O\(_2\). Since heterocysts do not have photosystem II, the induced expression of the *psaA* gene encoding the PSI reaction center protein under hyperbaric conditions suggests that PSI might play an role in the recovery/protection of nitrogenase activity under hyperbaric O\(_2\) conditions. However, further studies are obviously required. Of prime importance will be the generation of mutants where the genes identified in this study are inactivated in *Anabaena* strains capable of genetic transfer. Then, it may be possible to determine the precise role of these genes in the protection/recovery of nitrogenase activity in cultures incubated under hyperbaric O\(_2\) atmosphere.
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