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# High $CO_2$ Concentration Alleviates the Block in Photosynthetic Electron Transport in an *ndhB*-Inactivated Mutant of *Synechococcus* sp. PCC 7942[1]

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**The high-concentration $CO_2$-requiring mutant N5 of *Synechococcus* sp. PCC 7942 was obtained by the insertion of a kanamycin-resistant gene at the *EcoRI* site, 12.4 kb upstream of *rbc*. The mutant is unable to accumulate inorganic carbon internally and exhibits very low apparent photosynthetic affinity for inorganic carbon but a photosynthetic $V_{max}$ similar to that of the wild type. Sequence and northern analyses showed that the insertion inactivated a gene highly homologous to *ndhB*, encoding subunit II of NADH dehydrogenase in *Synechocystis* sp. PCC 6803 (T. Ogawa [1991] Proc Natl Acad Sci USA 88: 4275–4279). When the mutant and the wild-type cells were exposed to 5% $CO_2$ in air, their photosynthetic electron transfer capabilities, as revealed by fluorescence and thermoluminescence measurements, were similar. On the other hand, a significant decrease in variable fluorescence was observed when the mutant (but not the wild-type) cells were exposed to low $CO_2$ under continuous light. The same treatment also resulted in a shift (from 38–27°C) in the temperature at which the maximal thermoluminescence emission signal was obtained in the mutant but not in the wild type. These results may indicate that subunit II of NADH dehydrogenase is essential for the functional operation of the photosynthetic electron transport in *Synechococcus* under low but not high levels of $CO_2$. We suggest that the inability to accumulate inorganic carbon under air conditions stems from disrupture of electron transport in this mutant.**

The cyanobacterial CCM (see Aizawa and Miyachi, 1986; Kaplan et al., 1990; Miller et al., 1990; Coleman, 1991; Kaplan et al., 1991; Raven, 1991; Badger and Price, 1992, for recent reviews) involves an active transport of Ci, activated and energized by light (Kaplan et al., 1987). It has been proposed that PSII activity is required for the activation and that energization is via PSI activity (Ogawa et al., 1985). Recent studies (Miller et al., 1988, 1991) demonstrated a strong correlation between the rates of Ci uptake and of fluorescence quenching when *Synechococcus* cells were provided with a range of Ci concentrations. These data indicate a strong effect of Ci uptake on the photosynthetic electron transport, but the underlying mechanisms are not understood.

High-concentration $CO_2$-requiring mutants are being used as a tool to study the physiological and molecular mechanisms involved in the operation of the CCM (Marcus et al., 1986; Ogawa et al., 1987; Schwarz et al., 1988; Friedberg et al., 1989; Price and Badger, 1989; Bedu et al., 1990; Kaplan et al., 1990; Ogawa, 1990; Coleman, 1991; Kaplan et al., 1991; Lieman-Hurwitz et al., 1991; Ogawa, 1991; Badger and Price, 1992; Ogawa, 1992; Price et al., 1992; Schwarz et al., 1992). These mutants are also being used to test predictions made by a quantitative model that ascribes a central role in the functioning of the CCM to the structural organization of the carboxysomes (Price and Badger, 1989; Reinhold et al., 1989; Reinhold et al., 1991; Schwarz et al., 1992). In the case of *Synechococcus* sp. PCC 7942, the lesions in all of the mutants (Kaplan et al., 1991) were mapped in the genomic region of *rbc*, the operon encoding the large and small subunits of Rubisco. Clustering of genes involved in a particular physiological activity such as the transport of sulfate has already been demonstrated in *Synechococcus* (Green et al., 1989).

Inactivation of *ndhB*, encoding subunit II of NADH dehydrogenase in *Synechocystis* PCC 6803, resulted in a reduced ability to accumulate Ci internally. Consequently, the mutant could only grow in the presence of a high concentration of $CO_2$ (Ogawa, 1991). In the present report we present certain physiological characteristics of a mutant of *Synechococcus* PCC 7942 obtained following inactivation of *ndhB*, located 12 kb upstream of *rbc*. It is suggested that NADH dehydrogenase is required for photosynthetic electron transport in the presence of low, but not of high, $CO_2$.

## MATERIALS AND METHODS

Cultures of *Synechococcus* sp. strain PCC 7942 and the mutant N5 obtained following a modification of this strain (see below) were grown in BG11 medium (Stanier et al., 1971) supplemented with 20 mM Hepes-NaOH (pH 8.0) and kanamycin (25 μg/mL, in the case of the mutant) in the presence of high (5% $CO_2$ in air) or low (air) levels of $CO_2$,

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Abbreviations: Ci, inorganic carbon; CCM, inorganic carbon-concentrating mechanism; $F_{max}$, fluorescence intensity in the presence of 5 μM DCMU; $F_o$, signal intensity at 2 ms when the shutter is completely open; $F_s$, steady-state signal in the absence of DCMU; $Km^r$, kanamycin resistance; ORF, open reading frame; $Q_A$, primary electron-accepting plastoquinone of PSII; $Q_B$, second electron-accepting plastoquinone of PSII.

as previously described (Marcus et al., 1986). The rate of growth was determined from the increase in $A_{730}$ using a Shimadzu recording spectrophotometer UV200. The rates of Ci uptake and of Ci-dependent $O_2$ exchange were measured by the filtering centrifugation and the $O_2$ electrode techniques, respectively, as described earlier (Kaplan et al., 1988). Genomic DNA was isolated as described elsewhere (Williams, 1988). Standard rDNA techniques (Sambrook et al., 1989) were used for Southern analysis and cloning. Analysis of the DNA sequence was performed with the Sequenase kit (United States Biochemical Corp.). Both strands were sequenced with no ambiguities. Synthetic oligonucleotides (Biotechnology General, Rehovot, Israel) were used in cases in which the subclones did not overlap.

The high-concentration $CO_2$-requiring $Km^r$ mutant N5 was obtained as follows: a genomic *Sal*I fragment (3.8 kb) located 10.1 to 13.9 kb upstream of *rbc* (Fig. 1) was subcloned from a genomic library of *Synechococcus* PCC 7942 (kindly provided by Dr. A. Grossman) into the *Sal*I site of a modified Bluescript SK plasmid. The latter was obtained following digestion of the plasmid with *Eco*RV and *Sma*I and blunt-end ligation. The *Pst*I and *Eco*RI sites were therefore removed. The 1.3-kb *Eco*RI fragment bearing *npt*II (encoding $Km^r$) from pUC71 (Friedberg et al., 1989) was inserted in the *Eco*RI site (originally located 12.4 kb upstream of *rbc*, see schematic map in Fig. 1). *Synechococcus* cells were then transformed with the resulting 3.5-kb *Eag*I fragment, and the cells were grown in liquid medium in the presence of high $CO_2$ concentrations for 24 h, spread on plates containing BG11 supplemented with 25 μg/mL of kanamycin, and grown under either high or low levels of $CO_2$. The transformation resulted in high $CO_2$-requiring, $Km^r$ mutants, and one of them, designated N5, was chosen for further analysis.

Confirmation that the $Km^r$ cartridge was inserted in the desired *Eco*RI site in mutant N5 was obtained by Southern analysis (see below) and by sequence analysis of the relevant genomic fragment from N5. To sequence the region where the $Km^r$ cartridge was inserted, the genomic DNA from N5 was digested with *Eag*I, and the fragments of 3.5 to 4.0 kb were isolated and ligated within the *Eag*I site in Bluescript SK. Competent cells of *Escherichia coli* (DH5α) were transformed with the recombinant plasmids obtained and then by selection on plates containing LB medium (see Sambrook et al., 1989) supplemented with kanamycin (50 μg/mL). The plasmid was then isolated from $Km^r$ colonies. Sequence analysis of this fragment with the aid of synthetic oligonucleotides homologous to the 5′ and to the 3′ ends of the $Km^r$ cartridge was used to verify the site of insertion of the *Km*$^r$ gene in the genome of N5.

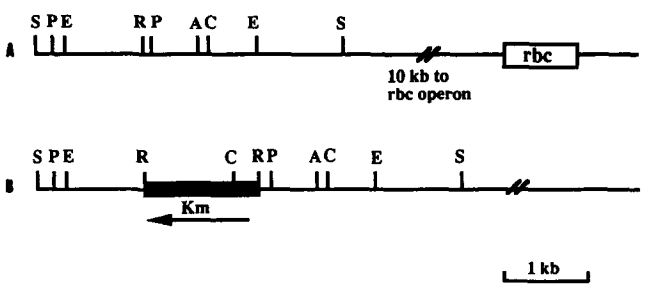


**Figure 1.** Schematic maps of the genomic region 10 kb upstream of the *rbc* operon of *Synechococcus* sp. PCC 7942 in the wild type (A) and the mutant N5 (B). Km, Kanamycin-resistance cartridge (the arrow indicates the orientation of the *Km*$^r$ gene); S, *Sal*I; P, *Pst*I; E, *Eag*I; C, *Cla*I; A, *Apa*I; R, *Eco*RI.

Total RNA was prepared from cells that were harvested in their exponential phase of growth. The cells were resuspended in 10 mM Tris, 1 mM EDTA (pH 8.0), extracted with an equal volume of phenol in the presence of 1% SDS and glass beads (212–300 μm, Sigma Chemical Co.), and then mixed on a vortex for 1 min with interim periods on ice. The cell extracts were centrifuged (4000*g* for 10 min), and the aqueous phase was reextracted with an equal volume of phenol:chloroform (1:1) and subsequently with chloroform. The nucleic acids were precipitated in the presence of 0.3 M sodium acetate and 70% ethanol. The pellet was resuspended in 10 mM Tris, 1 mM EDTA (pH 8.0), and the RNA was precipitated with 2 M LiCl overnight at 4°C. The precipitate was recovered by centrifugation, dried, and resuspended in a small volume of double-distilled water. Formaldehyde gel electrophoresis, blotting of the RNA, and hybridization with the DNA probe were performed as described elsewhere (Sambrook et al., 1989).

Fluorescence kinetics and thermoluminescence glow curves were recorded using high-concentration $CO_2$-grown cells (4 μg of Chl/mL in the former and 140 μg of Chl/mL in the latter). The cell suspensions were divided into two parts; one part was aerated for 1 h with 5% $CO_2$ in air, and the other was bubbled with air. The cells were then dark adapted for 5 min at room temperature, and the fluorescence kinetics or thermoluminescence glow curves were recorded. These measurements were also performed on cells that were illuminated (1500 μE $m^{-2}$ $s^{-1}$ of white light) for 2 min after the dark period.

Fluorescence induction was measured with the aid of a homemade fluorimeter connected to a PC computer, which operated the shutter and recorded the digitized signal as described previously (Ohad et al., 1988). Excitation (260 μE $m^{-2}$ $s^{-1}$) was provided by blue light (filter 4–96; Corning Glass, Corning, NY), and the photodiode was protected by a red cut-off filter (filter 6–65; Schott Glaswerke, Meinz, Germany). The signal was sampled at a rate of 15,000 points/s for the first 10 ms after the opening of the shutter and 100 to 200 points/s for the rest of the recording duration (2 s). The results are provided as $F_{max}$, $F_o$, and $F_s$. The ratios $(F_{max} - F_o)/F_o$ and $(F_{max} - F_s)/F_o$ were calculated because the former provides an indication of the capability of the reaction center of PSII to transfer electrons to $Q_A$, whereas the latter expresses the photosynthetic capability beyond the $Q_B$ site (Ohad and Hirschberg, 1992).

Thermoluminescence glow curves (Inoue, 1987) were recorded using a homemade apparatus. The cells (400 μL) were placed on the cell holder of the thermoluminescence apparatus and dark adapted for 5 min at room temperature. One to six saturating single-turnover light flashes were provided, followed by immediate cooling by liquid nitrogen. Charge recombination was elicited by heating the sample (0.7°C/s). The intensity of the light emitted resulting from charge recombination between the $Q_A^-$ or $Q_B^-$ and the S-states as a

function of the temperature during the heating was recorded by a photon counter connected to a PC computer programmed to control the heating rate and to display the results.

## RESULTS

### Southern, Northern, and Sequence Analyses

Southern analyses (Fig. 2) were performed on genomic DNA isolated from the wild type and from mutant N5, digested with *Pst*I, *Eag*I, and *Cla*I using the 1.3-kb *Eco*RI fragment (bearing *npt*II) and the genomic 1.3 kb *Pst*I fragment (Fig. 1) as probes. The *npt*II probe hybridized only with DNA fragments from the mutant, exhibiting bands of approximately 1.3 kb (*Pst*I), 4.2 kb (*Eag*I), and 4.9 and 1.0 kb (*Cla*I). The genomic *Pst*I probe hybridized with fragments of 1.3 kb (*Pst*I), 2.7 kb (*Eag*I), and 4.2 kb (*Cla*I) in DNA from the wild type and with fragments of 1.3 kb (*Pst*I), 4.3 kb (*Eag*I), and 5.0 kb (*Cla*I) in DNA from the mutant. The expected 1.0-kb fragment in the *Cla*I digest of the mutant was not detected, presumably because the homology with the probe extends over only 60 bp (Figs. 2 and 3). Hybridization with a probe prepared from the Bluescript SK plasmid did not detect any homologous fragment in the mutant, confirming that no plasmid sequences were present (not shown). These data clearly indicated that the cloned fragment had been inserted into the desired *Eco*RI site via a double-crossover recombination event and that complete segregation (i.e. replacement of the original with the modified region) was obtained. The insertion of the Km$^r$ cassette at the *Eco*RI site was also confirmed by sequence analysis of the relevant genomic DNA fragment cloned from N5 (not shown).

Sequence analysis of 2094 nucleotides between the *Cla*I site and the upstream *Sal*I site (Fig. 1) of the genomic DNA from the wild type (Fig. 3) revealed an ORF on the strand complementary to the one encoding *rbc*, starting from position 336 and extending to a stop codon in position 1898 (from the *Cla*I site). The *Eco*RI restriction site, where the Km$^r$ cartridge was inserted in mutant N5, is located within the ORF (position 666 from the *Cla*I site) and is underlined in Figure 3. A putative ribosome-binding site sequence (GAG) was found 15 bp upstream of the first ATG codon of the ORF.

Northern analyses with the aid of the 1.3-kb *Pst*I genomic fragment (containing most of the ORF) as a probe were

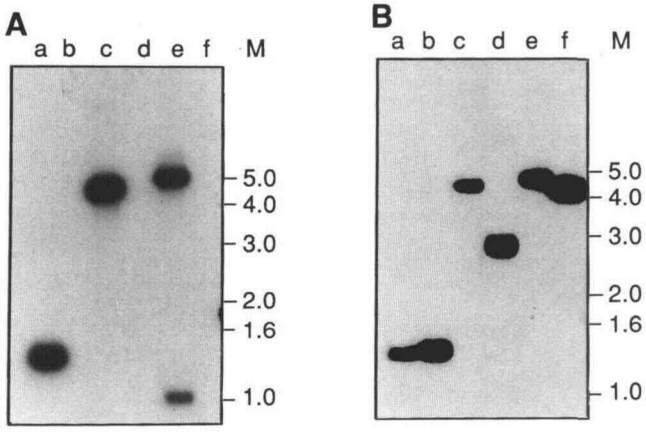


**Figure 2.** Southern analysis of the genomic DNA from mutant N5 and the wild type. A 1.3-kb *Eco*RI fragment containing the gene encoding Km$^r$ and a 1.3-kb *Pst*I fragment from the genomic DNA containing the *Eco*RI site (see Fig. 1A) were used as probes in A and B, respectively. Genomic DNA from mutant N5 is in lanes a, c, and e; genomic DNA from wild type is in lanes b, d, and f. The restriction enzymes used were *Pst*I (a and b), *Eag*I (c and d), and *Cla*I (e and f). M, Molecular size markers in kb.

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   1  ATCGATGCTTCGACGCGGTAATCCTTCGGCAGGTAGTTGCGGATGGTGCGCGCCTTGGTG
  61  GGCGACTCGACGATGACGAGTTTGGGCATGGAGTCACGATTCTGCCCGATTGGCAGCTAG
 121  GGAAGTCAAAAAAAGCGATCGCAGGCGATCGAGAGGGCCCAACCCAAAGGGCTGATCAAT
 181  GGGTAACCTCCTCTTCCACTTATAGCCAAAGATGTGAAGTCTCCTGCAACTGCGATCGCG
 241  GAAGGATCAGGCTGAGAGGAAGGTAGAATCAGTGGCTGATGCCTCAGGTTACTCAATCAA
 301  CCTGCTGGCGTTCCCCAGTTCTGAGTCGCACCGACCTATGGACTTTTTAACCCTCGCCGG
                        s.d                M  D  F  L  T  L  A  G
 361  TCAACTCAATGCCGGTGTGATTCTGCCAGAAGGCATCGTAATCGTCACACTGTTGACGGT
        Q  L  N  A  G  V  I  L  P  E  G  I  V  I  V  T  L  L  T  V
 421  TCTGGTCACGGATTTGATTTTGGGACGGCAATCGCTGCGCCTAACTCCAGCCCTCGCCAT
        L  V  T  D  L  I  L  G  R  Q  S  L  R  L  T  P  A  L  A  I
 481  CACGGGGCTTAGTGCTGCGATCGCAGTGCTCACACTCCAATGGAATACGAGTCAAAATCT
        T  G  L  S  A  A  I  A  V  L  T  L  Q  W  N  T  S  Q  N  L
 541  GGCTTTTCTTGGTGGCTTCAACGGCGATAATCTCAGTATTGTTTTCCGAGGCATTGTGCT
        A  F  L  G  G  F  N  G  D  N  L  S  I  V  F  R  G  I  V  L
 601  GCTGTCTGCAGCGGTCACCATCCTGCTCTCGATTCGCTACGTTGAGCAGTCGGGAACGTC
        L  S  A  A  V  T  I  L  L  S  I  R  Y  V  E  Q  S  G  T  S
 661  TCTTGGAGAATTCATTACGATTCTGCTCACGGCATCACTGGGGGGGATGTTTCTCTCCGG
        L  G  E  F  I  T  I  L  L  T  A  S  L  G  G  M  F  L  S  G
 721  TGCGAATGAGCTCGTAACTATTTTTGTGTCACTGGAAACGCTCAGTATTTCGTCTTATCT
        A  N  E  L  V  T  I  F  V  S  L  E  T  L  S  I  S  S  Y  L
 781  CTTGACGGGATATATGAAGCGCGACCCCCGTTCCAATGAAGCAGCACTGAAGTATTTGCT
        L  T  G  Y  M  K  R  D  P  R  S  N  E  A  A  L  K  Y  L  L
 841  GATTGGCGCTGCCAGCTCGGCTATCTTCCTCTACGGTGTTTCACTGCTCTACGGCTTAGC
        I  G  A  A  S  S  A  I  F  L  Y  G  V  S  L  L  Y  G  L  A
 901  TGGGGGGGAGACCCAGTTGCCTGCGATCGCGGAAAAACTGGGCGAAGCTCAGCCGCTGGC
        G  G  E  T  Q  L  P  A  I  A  E  K  L  G  E  A  Q  P  L  A
 961  ACTGCTGATTTCATTGATTTTTGTAATCGCTGGCATTGCTTTCAAAATTTCTGCGGTTCC
        L  L  I  S  L  I  F  V  I  A  G  I  A  F  K  I  S  A  V  P
1021  TTTCCACCAATGGACTCCGGACGTTTACGAAGGCTCGCCCACCCCGATCGTGGCGTTTCT
        F  H  Q  W  T  P  D  V  Y  E  G  S  P  T  P  I  V  A  F  L
1081  CTCGGTCGGTTCCAAAGCAGCGGGTTTTGCTTTGGCGATTCGCTTGCTGGTCACCGCCTA
        S  V  G  S  K  A  A  G  F  A  L  A  I  R  L  L  V  T  A  Y
1141  TCCGGCTTTGACCGAGCAATGGCACTTTGTCTTCACGGCCCTGGCCATCCTCAGCTTGGT
        P  A  L  T  E  Q  W  H  F  V  F  T  A  L  A  I  L  S  L  V
1201  GCTCGGTAACGTCGTGGCGCTCGCGCAAACCAGCATGAAGCGCCTGCTAGCCTACTCCTC
        L  G  N  V  V  A  L  A  Q  T  S  M  K  R  L  L  A  Y  S  S
1261  GATCGCCCAAGCTGGTTTTGTGATGATTGGCCTGATCGCTGGCACCGAAGCAGGCTATTC
        I  A  Q  A  G  F  V  M  I  G  L  I  A  G  T  E  A  G  Y  S
1321  CAGCATGGTTTATTACCTGCTGATTTACCTGTTTATGAATCTGGGTGGCTTTGCCTGTGT
        S  M  V  Y  Y  L  L  I  Y  L  F  M  N  L  G  G  F  A  C  V
1381  CATCCTCTTCTCGCTACGGACAGGAACGGACCAAATCAGCGAATACGCTGGCCTCTACCA
        I  L  F  S  L  R  T  G  T  D  Q  I  S  E  Y  A  G  L  Y  Q
1441  AAAAGATCCGCTGGTCACCCTCGGTCTGAGCCTCTGCCTGCTCTCGCTGGGCGGCATTCC
        K  D  P  L  V  T  L  G  L  S  L  C  L  L  S  L  G  G  I  P
1501  GCCGCTGGCTGGATTCTTTGGCAAGCTCTACCTGTTCTGGGCAGGTTGGCAGGCCGGGCT
        P  L  A  G  F  F  G  K  L  Y  L  F  W  A  G  W  Q  A  G  L
1561  GTATGGGTTGGTGTTGCTAGCGCTGATCACCAGTGTCATCTCGATCTACTACTACATCCG
        Y  G  L  V  L  L  A  L  I  T  S  V  I  S  I  Y  Y  Y  I  R
1621  TGTGATCAAGATGATGGTGGTCAAAGAGCCACAGGAGATGTCGGAGTCAGTGCGCAACTA
        V  I  K  M  M  V  V  K  E  P  Q  E  M  S  E  S  V  R  N  Y
1681  CCCCGAAACCAACTGGAATCTGCCGGGCATGCAGCCGTTGCGTGCTGGTTTGGTGGTTTG
        P  E  T  N  W  N  L  P  G  M  Q  P  L  R  A  G  L  V  V  C
1741  CGTGATTGCCACGGCCGTAGCAGGTATCCTCTCCAACCCACTGTTTAACCTTGCTAGTGC
        V  I  A  T  A  V  A  G  I  L  S  N  P  L  F  N  L  A  S  A
1801  TTCGGTGAGCGGCAGTTCCTTCCTGGGTTTAGCTCCTGCTGCAGAAGTGGTGACCACGAC
        S  V  S  G  S  S  F  L  G  L  A  P  A  A  E  V  V  T  T  T
1861  GGCAACCCCCGTCGCTCTGTCAGAACCGCCAGCTGCCAGTTAGCTAAACCGACCGCGATC
        A  T  P  V  A  L  S  E  P  P  A  A  S  *
1921  GCGGCTCACTCACCCCATCCTTTCCCAAGACAGCGCTAGCATGGCGCTGTCTTTCTTTTT
1981  GCAACCCCTTGTCTCTGCATGGCAGAATCCGCGCCTCCCGTCATTCAGTTCGAGCATGTC
2041  AGCAAGATCTACGGCGAAGGGGAAACTACGGTCCGTGCCTTGGATCATGTCGAC  2094
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**Figure 3.** Nucleotide sequence of the wild-type DNA between the *Cla*I site and the upstream *Sal*I site and the deduced amino acid sequence of the protein encoded by *ndhB*. The *Eco*RI site (position 668–673) where the *Km$^r$* gene was inserted is underlined. A putative −10 box (position 264–269) is underlined, and a possible ribosome-binding site (position 323–325) is labeled s.d and underlined. The inverted repeat (positions 1947–1956 and 1964–1973) in the downstream region of *ndhB* is also underlined.

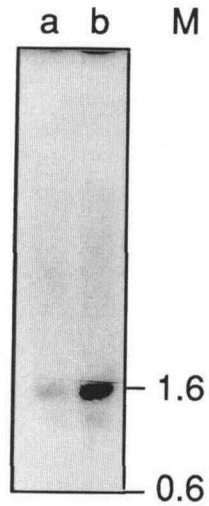


**Figure 4.** Northern analysis of total RNA from the wild type. A 1.3-kb *PstI* fragment containing most of *ndhB* was used as a probe. Lane a, Cells grown under high $CO_2$; lane b, cells transferred to low $CO_2$ 90 min before the isolation of the RNA. M, RNA markers in kb.

performed on RNA isolated from the wild type (Fig. 4) and the mutant (not shown, since we could not detect a transcript). The probe hybridized to a band of RNA of about 1.6 kb. The transcript was more abundant in RNA isolated from high $CO_2$-grown cells that were exposed to low $CO_2$ (air) for 90 min than in cells that were kept under high $CO_2$. These data may indicate transcriptional regulation of the ORF by ambient $CO_2$, but the mechanisms involved are not known. The initiation and termination points of the RNA were not determined, but analysis of the sequence in Figure 3 indicated a putative −10 sequence in position 264 (Schneider et al., 1991). The inverted repeat downstream of the ORF (underlined in Fig. 3) may be involved in transcription termination.

The ORF encodes a highly hydrophobic protein of 521 amino acids with an estimated molecular mass of 55,067 D. Sequence comparison (Fig. 5) showed 71.2% identity with the gene product of *ndhB* encoding subunit II of NADH dehydrogenase from *Synechocystis* sp. PCC 6803 (Ogawa, 1991). The analysis also indicated high homology to the relevant genes from the chloroplast genome of *Marchantia polymorpha* and human mitochondria, as was earlier reported by Ogawa (1991). Therefore, we concluded that the ORF is the *Synechococcus* equivalent of *ndhB*.

### Physiological Characterization of Mutant N5

The mutant N5 was unable to grow in the presence of air levels of $CO_2$ in either solid or liquid BG11 medium. In the presence of 5% $CO_2$, on the other hand, the rate of growth exhibited by the mutant was identical with that of the wild type. A short lag was observed in the growth rate of N5 in the presence of kanamycin (25 μg/mL), but the lag could be diminished when kanamycin was provided 24 h after the beginning of the growth experiment. The curves relating the rate of photosynthetic $O_2$ evolution by N5 as a function of the extracellular Ci concentration showed a photosynthetic $V_{max}$ similar to that of the wild type (320 μmol of $O_2$ $mg^{-1}$ of Chl $h^{-1}$) but approximately 100-fold lower apparent photosynthetic affinity for extracellular Ci. [$K_{½}$(Ci) was 0.1 and 10 mM Ci in the wild type and the mutant, respectively.] The detailed analyses of the growth curves and of the response of photosynthetic rate to the extracellular Ci concentration are not presented, because the data were similar to those reported in the case of other high $CO_2$-requiring mutants of *Synechococcus* (Marcus et al., 1986; Schwarz et al., 1988; Price and Badger, 1989; Lieman-Hurwitz et al., 1991). The low apparent photosynthetic affinity for Ci in mutant N5 is most probably attributable to the lack of accumulation of Ci internally (Table I). The Ci uptake activity of high $CO_2$-grown wild-type cells was rather low, but it increased following exposure to air levels of $CO_2$. On the other hand, N5 cells grown in the presence of high $CO_2$ and exposed to low $CO_2$ conditions showed very little activity of Ci uptake and consequently a relatively low internal Ci pool.

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A  MDFLTL-AGQLNAGVILPEGIVIVTLLTVLVTDLIL
B  ***SSNV*A*****T***********L**IV***G

   GRQSLRLTPALAITGLSAAIAVLTLQWNTSQNLAFL
   **KVALAL*Y***A**LVSVGL*VTS*SMADPIG*I

   GGFNGDNLSIVFRGIVLLSAAVTILLSIRYVEQSGT
   *A********I**A*IA**TV****M*V***Q*T**

   SLGEFITILLTASLGGMFLSGANELVTIFVSLETLS
   **A***A*****T*******A*****MV*I***M**

   ISSYLLTGYMKRDPRSNEAALKYLLIGAASSAIFLY
   *****M**********************S*******

   GVSLLYGLAGGETQLPAIAEKLGEA----QPLALLI
   *L******S******VL*****VN*DTVG*S*G*A*

   SLIFVIAGIAFKISAVPFHQWTPDVYEGSPTPIVAF
   A*V*******************************V***

   LSVGSKAAGFALAIRLLVTAYPALTEQWHFVFTALA
   **********V********FGGI*DE**VI*****

   ILSLVLGNVVALAQTSMKRLLAYSSIAQAGFVMIGL
   V**M***************M******G*********

   IAGTEAGYSSMVYYLLIYLFMNLGGFACVILFSLRT
   V**S*A**A***F*M*********A*S*I***T***

   GTDQISEYAGLYQKDPLVTLGLSLCLLSLGGIPPLA
   *S****D*****H****L*****I***********

   GFFGKLYLFWAGWQAGLYGLVLLALITSVISIYYYI
   *****I*I******SR*******G*V***V******

   RVIKMMVVKEPQEMSESVRNYPETNWNLPGMQPLRA
   **V*************VIK***AIK******R**QV

   GLVVCVIATAVAGILSNPLFNLASASVSGSSFLGLA
   *I*ATLV**SL****A*******TD**VSTKM*QT*

   PAAEVVTTTATPVALSEPPAASX
   LQQTGE*PAIAISHDLPX
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**Figure 5.** Alignment of the deduced amino acid sequence of *ndhB* from *Synechococcus* PCC 7942 (A) with the putative *ndhB* product of *Synechocystis* PCC 6803 (B) from Ogawa (1991). Identical amino acids are marked by asterisks (*); the termination codon is marked by X.

**Table I.** *Ci accumulation by wild-type (WT) and N5 cells of Synechococcus PCC 7942*

All of the cells were grown in the presence of high $CO_2$; some were then exposed to low $CO_2$ for 10 h. The cells were harvested and resuspended in 20 mM Hepes-NaOH, pH 8.0 (final cell density, 20 μg of Chl/mL). The cells were aerated for 30 min and then transferred to a microfuge tube where they were illuminated for 30 s, after which a mixture of $^{14}Ci$ and unlabeled Ci was provided to the indicated concentration. The experiments were terminated after 20 s by filtering centrifugation.

| [Ci] | Ci Accumulation | | | |
|---|---|---|---|---|
| | High $CO_2$ | | Low $CO_2$ | |
| | WT | N5 | WT | N5 |
| mM | nmol of Ci/μg of Chl | | | |
| 0.38 | 0.028 | 0.011 | 0.173 | 0.007 |
| 4.00 | 0.966 | 0.344 | 1.616 | 0.236 |

Uptake of Ci in cyanobacteria is activated and energized by photosynthetic light (see introduction). The lower Ci transport activity in mutant N5, a consequence of the inactivation of *ndhB*, might, therefore, be due to a defect in the activation or energization of the process. Fluorescence and thermoluminescence measurements were used to assess the effect of an ambient concentration of $CO_2$ on the photosynthetic electron transport capabilities (Figs. 6 and 7). The fluorescence parameters $F_o$, $F_{max}$, and $F_s$ and the ratios $(F_{max} - F_o)/F_o$ and $(F_{max} - F_s)/F_o$ calculated therefrom were rather similar

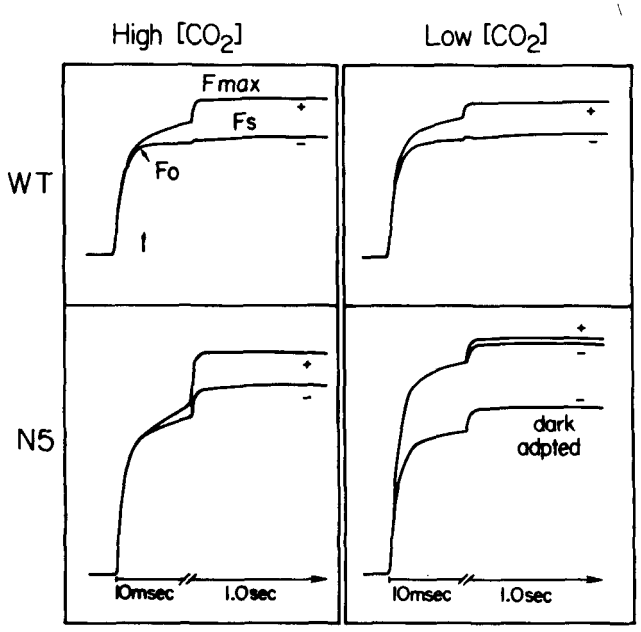


**Figure 6.** The fluorescence intensity of wild-type (WT) and N5 cells exposed to high or low levels of $CO_2$ in the presence (+) or absence (−) of DCMU (5 μM). The cells were dark adapted for 5 min at room temperature, and the first measurement was taken following a single flash (dark adapted). The cells were then kept in the light for 2 min, and the data provided are those obtained following the light treatment. (See "Materials and Methods" for experimental details.)

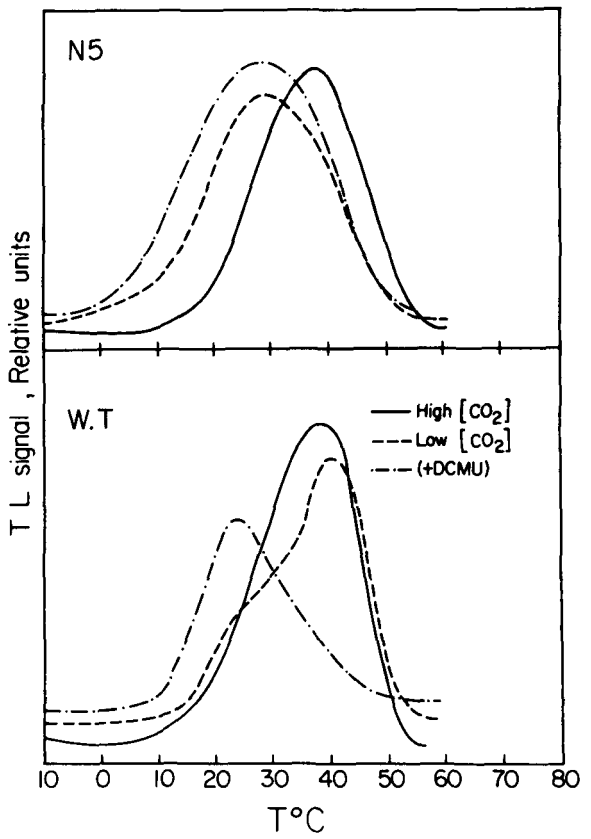


**Figure 7.** Thermoluminescence signal of wild-type (WT) and N5 cells exposed to high or low levels of $CO_2$. The signal was also recorded following exposure to 5 μM DCMU. (See "Materials and Methods" for experimental details.)

in wild-type and mutant cells grown at and exposed to high $CO_2$ (Fig. 6). These results were expected, because in the presence of high $CO_2$ the growth rates of the wild type and the mutant were similar. On the other hand, when N5 cells were exposed to low $CO_2$ the steady-state fluorescence intensity in the presence ($F_{max}$) and absence ($F_s$) of DCMU were almost identical. Therefore, although the ratio $(F_{max} - F_o)/F_o$ (which indicates the ability to transfer electrons to the $Q_A$ site) was hardly affected by exposure to low $CO_2$, the $(F_{max} - F_s)/F_o$ ratio decreased from 0.47 and 0.45 in wild-type and mutant cells exposed to high $CO_2$, respectively, to 0.30 and 0.05 in wild-type and mutant cells exposed to low $CO_2$, respectively. These data indicate that the mutant is essentially unable to transfer electrons in PSII beyond the $Q_B$ site under low $CO_2$ conditions and continuous light.

The conclusion that the inactivation of *ndhB* in mutant N5 resulted in inhibition of photosynthetic electron transport under low, but not under high $CO_2$ was further supported by the analysis of the thermoluminescence signal (Fig. 7). When wild-type and mutant cells were exposed to high $CO_2$, the maximum emissions were obtained at 37 to 39°C (in different experiments). Treatment with DCMU (5 μM), conditions under which the $Q_A$ signal is observed instead of that of $Q_B$, resulted in a shift of the maximal signals to 24 and 27°C in the wild type and N5, respectively. Exposure of wild-

type cells to low $CO_2$ did not affect the temperature of maximum emission. On the other hand, the same treatment resulted in a shift of the maximal signal to 27°C in the cases of the mutant. This shift was completely reversible following transfer of the cells back to high $CO_2$. When the wild-type cells were exposed to a series of flashes, they exhibited the well-established pattern (Inoue, 1987) of maximum emission after the second and sixth flash (not shown). This was not the case in N5 cells exposed to low $CO_2$, in which a clear, repeatable pattern was not observable. These data indicate that, in the mutant N5, the photosynthetic electron transport in PSII is severely inhibited following reduction in the ambient $CO_2$ concentration.

Inactivation of *ndhB* in mutant N5 resulted in 50 to 70% inhibition of the rate of dark respiration. Unlike the case of photosynthetic electron transport, the inhibition of dark respiration was not affected by the concentration of $CO_2$. However, because of the release of $CO_2$ in respiration, it was difficult to control its concentration in these experiments. Inhibition of dark respiration was also reported in the case of *Synechocystic* PCC 6803 and *Synechococcus* PCC 7002 mutants in which *ndhB* (Ogawa, 1991) or *ndhF* (Yu et al., 1993), respectively, were inactivated.

## DISCUSSION

Insertion of the $Km^r$ cartridge within an ORF that is highly homologous to *ndhB* (encoding subunit II of NADH dehydrogenase from *Synechocystis* PCC 6803 [Ogawa, 1991]) inactivated the gene and resulted in a high $CO_2$-requiring mutant, N5. It has been proposed (Ogawa, 1991, 1992) that this phenotype stems from a defect in the mutant's ability to accumulate Ci internally, as also suggested by the data presented in Table I. Consequently, there was a 100-fold reduction of the apparent photosynthetic affinity for extracellular Ci, which was too low to enable growth of the mutant under air levels of $CO_2$.

This is the first report of a high $CO_2$-requiring mutant of *Synechococcus* in which inability to concentrate Ci has been observed. The lesion in all of the other mutants that exhibit the same phenotype is due to either defective carboxysomes (Friedberg et al., 1989; Price and Badger, 1989; Price et al., 1992) or inability to produce purines following exposure to a low concentration of $CO_2$ (Schwarz et al., 1992). The RKa and M55 mutants of *Synechocystis* sp. PCC 6803, in which the *ndhB* was inactivated (Ogawa, 1991), were also unable to accumulate Ci internally.

Earlier studies demonstrated that uptake of Ci by cyanobacteria is light dependent and that photosynthetic light is involved in the activation and energization of the Ci-transporting system (Kaplan et al., 1987). Fluorescence and thermoluminescence measurements (Figs. 6 and 7) indicated severe inhibition of the photosynthetic electron transport capability beyond the $Q_B$ site in the mutant cells exposed to low $CO_2$. Transfer of the cells from low to high $CO_2$ alleviated the inhibition. Therefore, we conclude that the high $CO_2$-requiring phenotype of the mutant most probably results from the block in photosynthetic and respiratory electron transport observable when the cells are exposed to low $CO_2$. The reduced ability to accumulate Ci internally is likely to stem from the low-energy state of these cells consequent on this inhibition.

Studies by Myers (1993) have indicated that exposure of *Synechococcus* 6301 to a light of 680 nm (which is absorbed primarily by PSI) diverted the respiratory electron flow from oxygen reduction to $P700^+$. The role of subunit II of NADH dehydrogenase in cyanobacterial photosynthetic and respiratory electron transport and its possible involvement in the interaction between these processes (Peschek, 1987), however, are not yet understood. Furthermore, there are contradictory reports regarding the location of the subunits of NADH dehydrogenase. Ogawa (1992) suggested that they are located in the thylakoid membranes, whereas Berger et al. (1991) indicated both the thylakoid and cytoplasmic membranes as their site. Therefore, in a scheme proposed by Nicholls et al. (1992), the presence of two distinct electron transfer carrier systems, including NAD(P)H dehydrogenase, on both the cytoplasmic and thylakoid membranes was suggested.

Measurements of the rate of rereduction of $P700^+$ in the presence of different electron transport inhibitors and acceptors in whole cells of *Synechococcus* PCC 7002 and the *psaE*, *ndhF*, and *psaE/ndhF* mutants thereof (Yu et al., 1993) suggested that NADH dehydrogenase donates electrons to the plastoquinone pool, providing the link between the photosynthetic and respiratory electron transport in cyanobacteria. The scheme presented by these authors is supported by our observation that the photosynthetic electron transport beyond the $Q_B$ site is severely inhibited in N5 exposed to low $CO_2$. Further studies are, however, required to establish the means by which the presence of high $CO_2$ overcomes this inhibition. This may have significance for the elucidation of the well-established effect of bicarbonate on the activity of PSII (Eaton-Ray et al., 1986) and the possible involvement of NADH dehydrogenase in this effect.

Cloning of several genes encoding different subunits of the NADH dehydrogenase complex (Ogawa, 1992; Steinmuller, 1992) might help to elucidate the location and role of their gene products and the interaction between them. Inactivation of *ndhB*, *ndhK*, and *ndhL* (Ogawa, 1992), encoding different subunits of NADH dehydrogenase of *Synechocystis*, resulted in high $CO_2$-requiring mutants unable to accumulate Ci within the cell. On the other hand, mutants in which *ndhC* (Ogawa, 1992) and *ndhF* (Yu et al., 1993) were inactivated were able to grow in the presence of low $CO_2$. We have isolated a revertant of N5 that probably resulted from a suppression mutation as suggested by the fact that Southern analysis (not shown) gave similar results to those obtained in N5. Analysis of such mutants might help to elucidate the role of, and the interaction between, the different subunits of NADH dehydrogenase.

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