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Mutation of *ndh* Genes Leads to Inhibition of CO_2 Uptake Rather than HCO_3^- Uptake in *Synechocystis* sp. Strain PCC 6803

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Six mutants (B1 to B6) that grew poorly in air on BG11 agar plates buffered at pH 8.0 were rescued after mutations were introduced into *ndhB* of wild-type (WT) *Synechocystis* sp. strain PCC 6803. In these mutants and a mutant (M55) lacking *ndhB*, CO_2 uptake was much more strongly inhibited than HCO_3^- uptake, i.e., the activities of CO_2 and HCO_3^- uptake in B1 were 9 and 85% of those in the WT, respectively. Most of the mutants grew very slowly or did not grow at all at pH 6.5 or 7.0 in air, and their ability to grow under these conditions was correlated with CO_2 uptake capacity. Detailed studies of B1 and M55 indicated that the mutants grew as fast as the WT in liquid at pH 8.0 under air, although they grew poorly on agar plates. The contribution of CO_2 uptake appears to be larger on solid medium. Five mutants were constructed by inactivating each of the five *ndhD* genes in *Synechocystis* sp. strain PCC 6803. The mutant lacking *ndhD3* grew much more slowly than the WT at pH 6.5 under 50 ppm CO_2, although other *ndhD* mutants grew like the WT under these conditions and showed low affinity for CO_2 uptake. These results indicated the presence of multiple NAD(P)H dehydrogenase type I complexes with specific roles.

In cyanobacteria the type I NAD(P)H dehydrogenase complex (NDH-1) is a proton-pumping complex that has been shown to be involved in both the respiratory and photosynthetic electron transport chains (25). NDH-1 acts as a plastoquinone oxidoreductase with NADH or NADPH as a substrate. There is evidence that NDH-1 is located in the cytoplasmic membrane as well as the thylakoid membrane, but evidence for location in the cytoplasmic membrane has not been consistent. NDH-1 is composed of 12 recognized subunits, with the large, hydrophobic NdhB, NdhD, and NdhF subunits being core membrane components. The availability of a whole-genome database for *Synechocystis* sp. strain PCC 6803 has shown that most *ndh* genes are present as single copies; however, *ndhD* and *ndhF* are present as multiple copies with five and four members, respectively (note that NdhF4 has homology with NdhD5 and could be counted as an NdhD homolog). There is a marked degree of protein sequence divergence within the NdhD and NdhF families, and this has led to suggestions that several NDH-1 complexes may exist in cyanobacteria, each with different NdhD and/or NdhF subunits and with each potential complex having differing functions (21, 24). One main role for NDH-1 in the thylakoid membrane is to participate in cyclic electron flow around photosystem I and to pump protons into the lumen, thereby contributing to ΔpH-driven ATP generation at the expense of NADPH (11–13). However, other roles for NDH-1 are possible.

It has been demonstrated that NDH-1 is essential for inorganic carbon (C_i) transport in cyanobacteria (4, 5, 10, 16–18, 21). Inactivation of *ndhB* or *ndhL* in *Synechocystis* sp. strain PCC 6803 greatly reduced the activities of CO_2 and HCO_3^- uptake, and in the past it has been assumed that the NDH-1-dependent cyclic electron transport supplies ATP to drive the C_i uptake (16–18). However, the question of why ATP produced by linear electron transport in these mutants does not drive the C_i transport, remains unresolved, and it was not certain whether ATP energizes the uptake of CO_2 and HCO_3^-. The finding that the *cmp* operon encodes an ATP binding cassette transporter for HCO_3^- clearly indicates the presence of at least one type of ATP-dependent HCO_3^- transporter (22). Li and Canvin (8) reported that HCO_3^- uptake is supported by linear electron transport while CO_2 uptake is supported by cyclic electron transport, based on the observation of differential effects of electron transport inhibitors and acceptors on uptake of the two carbon species. The results suggested that ATP produced by noncyclic electron transport energized HCO_3^- transport. Although the involvement of NDH-1 in CO_2 uptake is clearly demonstrated, little is known about the mechanism of CO_2 uptake and how it is energized.

Some *ndh* mutants, such as M55 (*ndhB*), show major inhibition of both CO_2 and HCO_3^- uptake (16–18), whereas other mutants, such K22 and A41 (*ndhD3*), show an effect largely on high-affinity CO_2 uptake (5). However, the previously described M55 mutant of *Synechocystis* sp. strain PCC 6803 is a highly disruptive insertional mutant of the single-copy *ndhB* gene (16–18), and it could be argued that removal of the NdhB protein could cause nonassembly of all NDH-1 complexes and production of secondary phenotypes. A less disruptive approach is to mutagenize *ndhB* with multiple single-base mutations and to select for mutants where the NdhD protein is still capable of assembly but lacks most functionality. To help understand the role of NDH-1 in C_i transport, we investigated whether random-site mutations within the *ndhB* gene of *Synechocystis* sp. strain PCC 6803 inhibit the uptake of CO_2 and HCO_3^- to the same extent. We show in this paper by measurement of CO_2 and HCO_3^- uptake in these mutants that CO_2 uptake is much more strongly inhibited than HCO_3^- uptake, a result more consistent with the effect of *ndhD3* mutations in *Synechococcus* sp. strain PCC 7002.

Synechocystis sp. strain PCC 6803 possesses five *ndhD* genes (3), with *sll1733* (designated *ndhD3*) being homologous to *ndhD3* in *Synechococcus* sp. strain PCC 7002. We describe in this paper the growth characteristics of mutants constructed by knocking out each of the *ndhD* genes in *Synechocystis* and show

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that *ndhD3* has a specific role in inducing high-affinity CO_2 uptake. Another purpose of the present work was to make some comparisons among the suite of *ndhD* mutants, as well as existing mutants such as M55 and new multiple-point mutants of *ndhB*, and attempt to provide a consensus view of the role of NDH-1 in C_i uptake in cyanobacteria.

MATERIALS AND METHODS

Growth conditions. WT and mutant cells of *Synechocystis* sp. strain PCC 6803 were grown at 30°C in BG11 medium (26) buffered with 20 mM *N*-Tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid (TES)-KOH (pH 8.0 and 7.0) or 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH (pH 6.5) and aeration with 3% (vol/vol) CO_2 in air or with air. The solid medium was BG11 supplemented with 1.5% agar, 5 mM sodium thiosulfate, and 20 mM the same buffer. When air containing 50 or 20 ppm CO_2 was used, the air was passed through Wako lime (Wako Co., Tokyo, Japan) packed in a metal tube (5-cm diameter and 20-cm length) at a flow rate of approximately 200 ml/min. Continuous illumination was provided by fluorescent lamps generating photosynthetically active radiation of 60 μmol of photons/m^2/s.

Construction of mutants. A clone, p*UC*EE5.1, derived from p*UC*EK11.8 (16), was used for random mutagenesis of *ndhB*. A kanamycin resistance cartridge (Km^r) cassette was inserted at the *Bgl*II site downstream of *ndhB*. Insertion of the cassette at this site did not change the phenotype of WT *Synechocystis* cells transformed with this construct (16). A fragment of 606 bp between the *Bam*HI and *Spe*I sites was removed from the *ndhB* gene, and fragments containing the same DNA region were synthesized by a PCR method that introduces random mutations (7) and then reinserted between the two restriction sites. The sequences upstream of the *Bam*HI site and downstream of the *Spe*I site were used to design the PCR primers, and p*UC*EE5.1 was used as a template. A library thus constructed, containing various mutated *ndhB* genes, was used to transform the WT cells of *Synechocystis* sp. strain PCC 6803 into Km^r mutants, using the protocol of Williams and Szalay (29). The transformants were spread on agar plates containing BG11 medium and kanamycin (10 μg/ml) buffered at pH 8.0, and the plates were incubated in air. Eight mutants were rescued as colonies that grew slowly under air. The mutated *ndhB* in the transformants was segregated to homogeneity (by successive-streak purification) as determined by PCR amplification. The *ndhB* gene in each mutant was amplified by PCR and used as a DNA-sequencing template.

M55 is the mutant constructed by inserting a Km^r cassette at the *Bam*HI site in *ndhB*, as described previously (16).

The *slr0331* (*ndhD1*), *slr1291* (*ndhD2*), *sll1733* (*ndhD3*), *sll0027* (*ndhD4*), *slr2007* (*ndhD5*), *sll1732* (*ndhF3*), and *sll1734* genes of *Synechocystis* sp. strain PCC 6803 (3) were amplified by PCR and then cloned into the pGEM-T vector (Promega, Madison, Wis.). The 801-bp *Bal*I/*Bal*I fragment in *ndhD1*, the 1,049-bp *Bal*I/*Bal*I fragment in *ndhD2*, the 51-bp *Eco*RV/*Kpn*I fragment in *ndhD3*, the 1,029-bp *Bal*I/*Bal*I fragment in *ndhD4*, the 878-bp *Nhe*I/*Stu*I fragment in *ndhD5*, the 336-bp *Eco*RI/*Pst*I fragment in *ndhF3*, and the 45-bp *Eco*RI/*Pst*I fragment in *sll1734* were replaced with cassettes that confer resistance to spectinomycin (Sp^r) for *ndhD1* mutants, chloramphenicol (Cm^r) for *ndhD2* mutants, kanamycin (Km^r) for *ndhD3*, *ndhD4*, *ndhF3*, and *sll1734* mutants, and hygromycin (Hyg^r) for *ndhD5* mutants. These cassettes were inserted in parallel or antiparallel to the direction of the genes. The constructs were used to transform the WT cells of *Synechocystis* sp. strain PCC 6803 to generate the *ndhD1*, *ndhD2*, *ndhD3*, *ndhD4*, *ndhD5*, *ndhF3*, and *sll1734* mutants. The segregation of inactivated genes in each mutant was determined by the method described above.

Silicone oil-filtering centrifugation. The uptake of $^{14}CO_2$ and $H^{14}CO_3^-$ was measured in high-CO_2-grown cells (aerated with air for 18 h in the light) using the silicone oil-filtering centrifugation method (28). The cells were suspended in BG11 medium buffered with 20 mM TES-KOH (pH 8.0) at a chlorophyll (Chl) concentration of 20 μg/ml. C_i uptake was initiated by the addition of $^{14}CO_2$ or $H^{14}CO_3^-$ in the light and terminated by centrifugation.

Determination of growth characteristics. WT and mutant strains grown under 3% CO_2 were collected and resuspended in fresh BG11 medium to optical densities at 730 nm (OD_{730}s) of 1.0, 0.1, and 0.01. Two microliters of the cell suspensions was spotted onto BG11 agar plates buffered at various pHs. The plates were incubated under 3% (vol/vol) CO_2 in air or under air for 5 days with continuous illumination by fluorescent lamps at a photosynthetically active radiation intensity of 60 μmol of photons/m^2/s. The OD_{730} was measured with a recording spectrophotometer, model UV2200 (Shimadzu Co., Kyoto, Japan).

Electrophoresis and Western analysis. An antibody was raised against partial NdhB fused to glutathione-*S*-transferase (GST). The *ndhB* gene amplified by PCR was digested with *Mvo*I and *Mba*I, and a fragment of 226 bp (from base 419 to 644 as numbered from the initiation codon of *ndhB*) was excised from a gel after electrophoresis of the digest. The fragment was blunted and ligated to the *Sma*I site of pUC119. The insert DNA was excised with *Eco*RI and *Bam*HI and was ligated to pGEX-3X (Pharmacia, Uppsala, Sweden). The construct was used to transform *Escherichia coli*. GST-NdhB (partial) formed inclusion bodies, which were isolated, solubilized with 5% sodium dodecyl sulfate (SDS), and electrophoresed by SDS-polyacrylamide gel electrophoresis. A prominent band of GST-NdhB (partial) at 33 kDa was cut out from the gels and mashed with a pestle and mortar to be injected into rabbits.

Total membrane fractions were prepared from the WT and mutant cells as described by Nilsson et al. (15). SDS-polyacrylamide gel electrophoresis was performed in the system of Laemmli (6). Polypeptides were electrotransferred to nitrocellulose membrane and reacted with the antibody against GST-NdhB (partial). Goat anti-rabbit immunoglobulin G conjugated to peroxidase was used as the second antibody, and reacting bands were detected with an ECL kit (Amersham).

RT-PCR analysis. RNA from air-grown *Synechocystis* cells was extracted by the method of Aiba et al. (1) and then subjected to reverse transcriptase (RT) PCR (14). Primers specific to the 3′ ends of *ndhF3*, *ndhD3*, *sll1734*, and *sll1735*, respectively, were used for the RT reaction. The subsequent PCR was performed with a pair of primers specific to *ndhF3*.

Mass spectrometric measurements. Measurements of the initial CO_2 uptake rate and kinetics for CO_2 and HCO_3^- uptake under steady-state conditions were performed according to the method of Badger et al. (2).

Other methods. Unless otherwise stated, standard techniques were used for DNA manipulation (9). Pigments in the cells were extracted in methanol, and the Chl concentration in the extract was determined (20).

TABLE 1. Substitution of amino-acid residues in NdhB of the B1-B6 mutants

Mutant	Substitution of amino acid residues[a]
B1	76N/Y, 104Q/R, 114I/V, 148Y/N, 149L/P, 151T/A, 197L/F, 241V/A
B2	104Q/R, 114I/V, 148Y/N, 149L/P, 151T/A, 197L/F, 241V/A
B3	76N/Y, 104Q/R, 114I/V, 161N/S, 166K/E, 193T/A
B4	148Y/N, 149L/P, 151T/A, 197L/F, 241V/A
B5	154M/T, 166K/E, 170I/V, 188L/S, 195L/M, 229I/V
B6	148Y/N, 166K/E

[a] Amino acid residues are expressed as letters. The amino acids were numbered from the N-terminal methionine residue. The letters with the numbers show the amino acids in the WT, and those after the shill indicate the residues in the mutants.

RESULTS

Isolation of *ndhB* mutants and sites of mutations. Eight mutants that grew poorly in air on agar plates buffered at pH 8.0 were rescued after mutations were introduced into *ndhB* of WT *Synechocystis* sp. strain PCC 6803 by low-fidelity PCR (7). Sequencing of impaired *ndhB* genes in these mutants (designated B1 to B8) revealed that B7 and B8 were identical to B1. Amino acid residues substituted in the B1 to B6 mutants are summarized in Table 1. There were substitutions of 2 to 8 amino acid residues in each of the *ndhB* mutants.

CO_2 and HCO_3^- uptake in the *ndhB* mutants. Figure 1 shows the time courses of CO_2 uptake (left panel) and HCO_3^- uptake (right panel) for WT, B1, and M55 cells grown at pH 8.0 under 3% CO_2 and then transferred to aeration with air for 18 h in the light. The activity of CO_2 uptake was very low in the B1 and M55 mutants, but the activity of HCO_3^- uptake in B1 was as high as that in the WT, whereas M55 possessed half the WT activity. High-CO_2-grown cells of these mutants also showed very low activity of CO_2 uptake, indicating that the low activity is not a result of aeration with air in the light (data not shown). Thus, CO_2 uptake was inhibited preferentially in these mutants. This was confirmed by the results summarized in Fig. 2, where the activities of CO_2 and HCO_3^- uptake in the *ndhB* mutants are shown as percentages of the WT activities. Although the extent of inhibition varied, CO_2 uptake was more strongly inhibited than HCO_3^- uptake in all the mutants examined. Thus, CO_2 uptake is strictly dependent on NDH-1 whereas HCO_3^- uptake proceeds even in the absence of this enzyme. However, the result was that HCO_3^- uptake was partly inhibited in the mutants, although the extent of inhibition was at most 50%, even in M55. This suggests that the low activity of HCO_3^- transport is a secondary effect caused by the

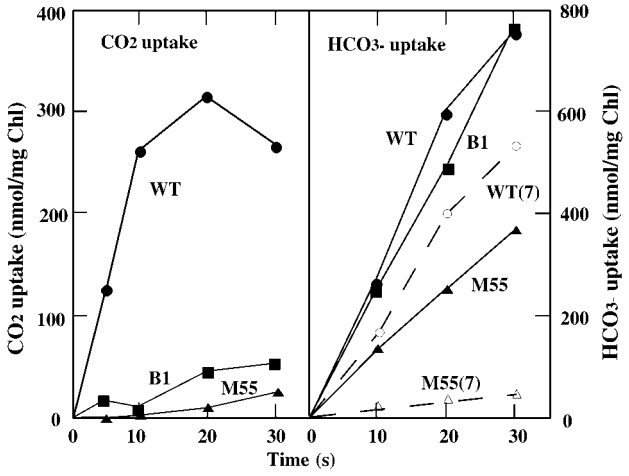

FIG. 1. Time courses of uptake of $^{14}CO_2$ (left panel) and $H^{14}CO_3^-$ (right panel) into WT (circles), B1 (squares), and M55 (triangles) cells, measured by the silicone oil-filtering centrifugation method. The cells were grown under 3% CO_2 and then aerated with air for 18 h in the light at pH 8.0 (solid curves) or pH 7.0 (dashed curves). The cells were suspended in BG11 medium containing 15 mM NaCl buffered with 20 mM HEPES-KOH, pH 8.0. The concentrations of $^{14}CO_2$ and $H^{14}CO_3^-$ were 7.9 and 61 μM, respectively.

absence of NDH-1 or that NDH-1 has a supplemental role in the transport of HCO_3^-.

The time courses of HCO_3^- uptake in WT and M55 cells grown initially at pH 7.0 under 3% CO_2 and then transferred to aeration with air are shown in Fig. 1. The result indicated that an increase in HCO_3^- uptake activity was not induced in M55 cells at this pH.

Growth characteristics of the *ndhB* mutants. To explore how the inhibition of CO_2 and HCO_3^- uptake affects the growth characteristics of the cells, growth of the mutant strains was examined on solid BG11 medium buffered at various pHs under 3% CO_2 or air. At pHs 8.0 and 7.0 under 3% CO_2, all the mutants except M55 grew as fast as the WT (Fig. 3A and B). M55 grew more slowly than the WT at pH 8.0 and was unable to grow at pH 6.5 under the high-CO_2 conditions (Fig. 3A to C). The B1 and B2 mutants grew more slowly at pH 6.5 in 3% CO_2 (Fig. 3C). All the mutants except B4 and B6 grew

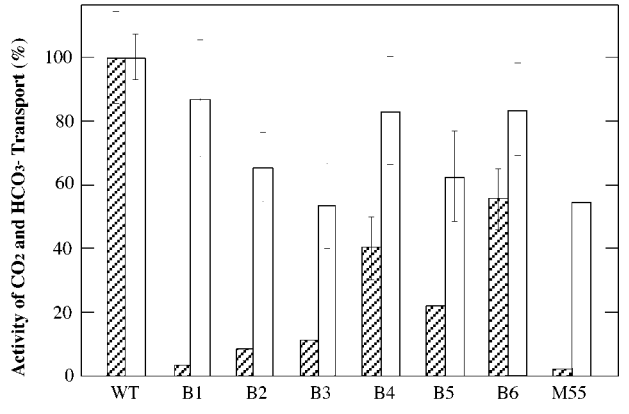

FIG. 2. Amount of C_i taken up by the mutant cells during incubation with $^{14}CO_2$ (hatched bars) for 10 s or with $H^{14}CO_3^-$ (open bars) for 20 s in the light. Each value is shown as a percentage of the value obtained for WT cells (259 nmol of CO_2/mg of Chl per 10 s and 473 nmol of HCO_3^-/mg of Chl per 20 s), and the error bars indicate standard deviations (n = 3). Cells grown at pH 8.0 were suspended in the same medium as for Fig. 1.

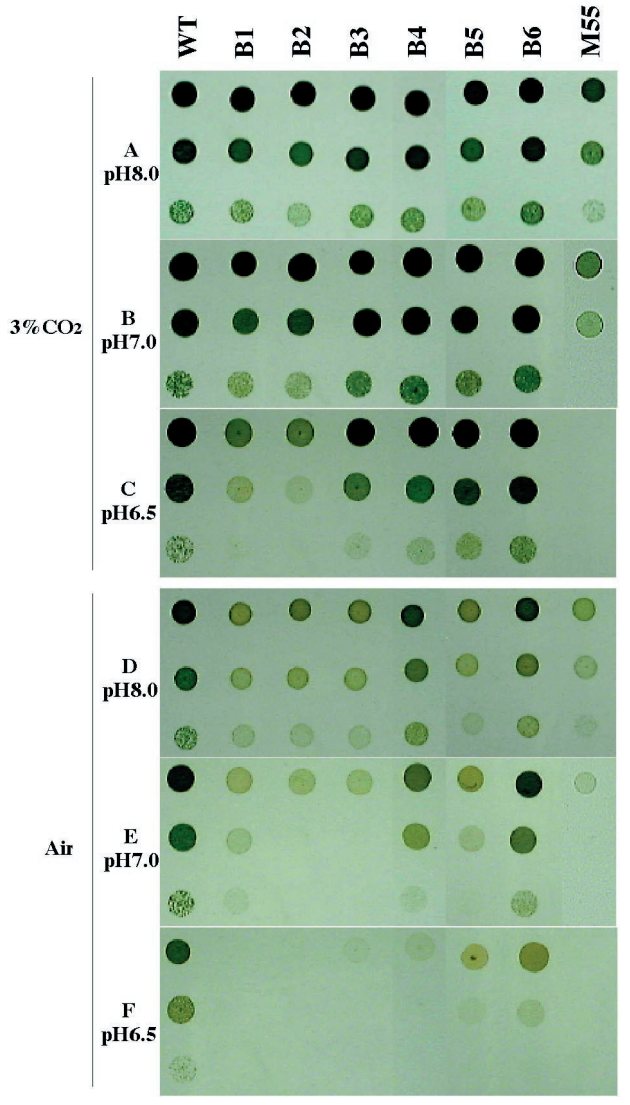

FIG. 3. Effects of pH and CO_2 concentration on the growth of the WT and mutants on agar plates. The WT and *ndhB* mutant cells of *Synechocystis* were pelleted by centrifugation and resuspended in BG11 medium, pH 8.0, 7.0, or 6.5. Two microliters of the cell suspensions, OD_{730} values of 1.0 (upper row of each panel), 0.1 (middle row), and 0.01 (lower row), were spotted on agar plates containing BG11 medium buffered at pH 8.0, pH 7.0, and pH 6.5. The plates were incubated under 3% (vol/vol) CO_2 in air (A to C) or under air (D to F) for 5 days at 60 μmol of photons of photosynthetically active radiation/m^2s^{-1}.

more slowly than the WT even at pH 8.0 under air (Fig. 3D) and very slowly at pH 7.0 (Fig. 3E). Most of the mutants were unable to grow at pH 6.5, and the growth of B6 was relatively poor under these conditions (Fig. 3F). The ability of the mutants to grow under atmospheric levels of CO_2, especially at pH 7.0 or 6.5, was correlated with their activity for CO_2 uptake (Fig. 2 and 3).

Figure 4 shows the growth rates of the WT, B1, and M55 cells in liquid medium plotted as a function of pH. There was not much effect of pH on growth rates of WT cells between pHs 8.0 and 6.5, either at 3% CO_2 or in air (left panel). In contrast, the growth of B1 and M55 in air was strongly dependent on the pH of the medium. Under air, both mutants grew as fast as the WT at pH 8.0, but the growth of B1 was very poor and M55 was unable to grow at pH 7.0 or 6.5 (middle and right

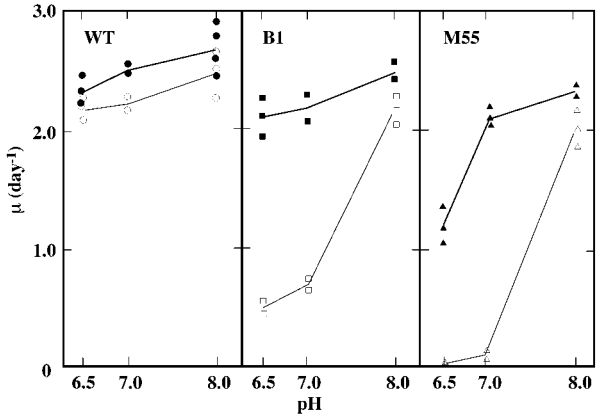

FIG. 4. Effect of pH on growth rates of WT, B1, and M55 in liquid BG11 medium during aeration with 3% (vol/vol) CO_2 in air (closed symbols) or under air (open symbols). Growth rates are shown by the μ values. Doubling time (in days), 0.693/μ.

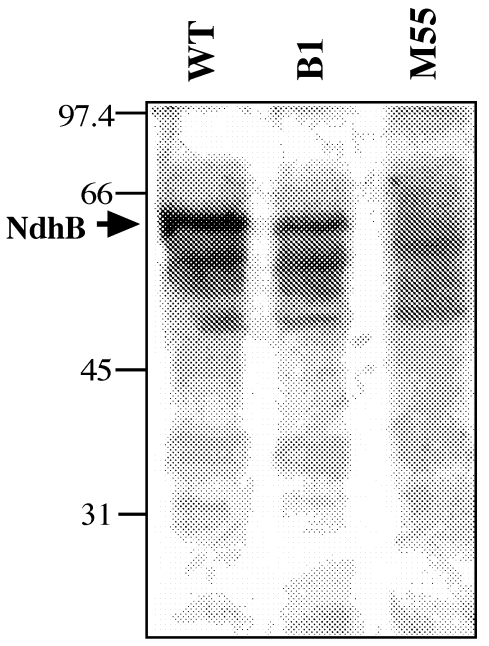

FIG. 5. Immunoblot of NdhB in the total cell membranes of *Synechocystis* sp. strain PCC 6803 WT, B1, and M55. Samples (10 μg of proteins) were solubilized at room temperature and boiled for several minutes and were run in a 10% gel. The antibody against GST-NdhB (partial) was used for immunoblotting.

panels). These results indicate that, in liquid, inorganic carbon is predominantly supplied by CO_2 uptake in WT cells in neutral- or acidic-pH media gassed with atmospheric levels of CO_2. The high growth rates of the mutants at pH 8.0 in liquid medium in air, in spite of their slow growth on agar plates under the same pH and CO_2 level, suggest that the contribution of HCO_3^- transport may be larger for growth in liquid medium than for growth on the surfaces of agar plates. The B1 mutant grew as fast as the WT under 3% CO_2 for the pH range between 8.0 and 6.5 (Fig. 4, middle panel). There was a large drop in the growth rate of M55 at pH 6.5 even under 3% CO_2 (right panel). It appears that NDH-1 has a role in the growth of cells at acidic pHs.

Identification of NdhB. The antibody raised against GST-NdhB (partial) cross-reacted with several bands in the total cell membranes of the WT but most strongly with the band at 55 kDa, the molecular mass of NdhB estimated from the DNA database translation (Fig. 5, left lane). No cross-reacting band was present at 55 kDa in the membranes of M55 (right lane), indicating that the cross-reacting band in the WT is NdhB. Western analysis with the total cell membranes of B1 using the same antibody gave a band at 55 kDa (Fig. 5, middle lane). The density of the band in B1 membranes was lower than that in the WT membrane. The results indicated that NDH-1 is present in the B1 mutant, although the protein may not be fully functional.

Growth of *ndhD* mutants under limiting CO_2 conditions. *ndhD1*, *ndhD2*, *ndhD3*, *ndhD4*, and *ndhD5* mutants grew as fast as the WT at pH 7.0 in air (data not shown). Measurement of the growth rates of these mutants at pH 6.5 and 50 ppm CO_2 revealed that the *ndhD3* mutant grew at about a third of the rate of WT cells, although the rest of the *ndhD* mutants showed virtually the same growth rate as the WT (Table 2). *ndhF3* and *sll1734* mutants constructed by inactivating the genes upstream and downstream of *ndhD3*, respectively, showed the same growth characteristics as the *ndhD3* mutant at pH 6.5 and 50 ppm CO_2, but inactivation of *sll1735* downstream of *sll1734* did not have a significant effect on the growth rate under these conditions. These results suggested that *ndhD3*, *ndhF3*, and *sll1734* have a common and specific effect on the growth of cells under limiting CO_2 conditions.

Expression of *ndhD3* and neighboring genes. The products of RT reactions with primers specific to the 3′ ends of *ndhF3*, *ndhD3*, *sll1734*, and *sll1735* were used as templates for the subsequent PCR with a set of primers specific to *ndhF3*. The PCR with three different templates gave the same products (Fig. 6, lanes 1 to 3). This indicated that *ndhF3*, *ndhD3*, and *sll1734* are expressed together as an operon. No PCR product was found when a primer specific for *sll1735* was used for the RT reaction (lane 4), indicating that the gene is expressed independently of the upstream genes.

Initial and steady-state rates of CO_2 uptake. Cyanobacteria exhibit a transient and rapid uptake of CO_2 following a dark-to-light transition. The CO_2 uptake during steady-state photosynthesis and the relative affinity for gross CO_2 uptake can be measured and calculated according to a method developed by Badger et al. (2). Using this method, the steady-state rate and initial rate of CO_2 uptake were measured and calculated under various C_i concentrations, allowing $K_{0.5}(CO_2)$, the concentration of CO_2 required to reach the half-maximal uptake rate, to be determined. Figure 7 shows the $K_{0.5}(CO_2)$ and V_{max} values

TABLE 2. Growth rates of WT and mutants of *Synechocystis* sp. strain PCC 6803[a]

Cells	μ (day^{-1})
WT	1.06 ± 0.15
ndhD1	1.12 ± 0.06
ndhD2	1.05 ± 0.02
ndhD3 (*sll1733*)	0.35 ± 0.03
ndhD4	0.95 ± 0.20
ndhD5	0.90 ± 0.11
ndhF3 (*sll1732*)	0.36 ± 0.03
sll1734	0.37 ± 0.02
sll1735	0.90 ± 0.21

[a] WT and mutant cells were grown at 30°C in BG11 medium with 20 mM MES-KOH (pH 6.5) under aeration with 50 ppm CO_2 at a light intensity of 60 μmol PAR/m^2/s. Specific growth rates (μ) are expressed per day with standard deviations (n = 3). To convert the growth rates to doublings per day, divide ln 2(0.693) by μ.

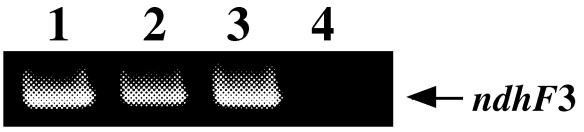

FIG. 6. RT-PCR analysis of total RNA from *Synechocystis* cells showing the expression of *ndhF3*, *ndhD3*, and *sll1734* as an operon. RNA was prepared from cells grown at pH 8.0 under 50 ppm CO_2. PCR amplification was performed with a set of primers specific to *ndhF3* (ATTATCTGGCTAGTACC and GAATAG CTAAGAAAGGC), and the product was 1.8 kbp. The templates used for PCR were cDNAs which were synthesized by reverse transcription of mRNA by addition of RT with primers specific to *ndhF3* (lane 1), *ndhD3* (lane 2), *sll1734* (lane 3), and *sll1735* (lane 4), respectively.

for the steady-state (Fig. 7A) and initial (Fig. 7B) rates of CO_2 uptake obtained for the WT and *ndhD3* mutant cells grown at 2% CO_2 or induced overnight at 20 ppm CO_2. There was no significant difference between the V_{max} values of the WT and mutant cells. In contrast, the $K_{0.5}(CO_2)$ values for initial and steady-state CO_2 uptake were significantly higher in the mutant than in the WT, in cells both before and after 18 h of induction at 20 ppm CO_2. The affinity of steady-state CO_2 uptake for CO_2 did not change in the mutant after induction at 20 ppm CO_2, while the affinity increased about 2.5-fold in the WT after induction. Thus, *ndhD3* appears to be essential for the induction of high-affinity CO_2 uptake. In *Synechocystis* sp. strain PCC 6803, the V_{max} of net CO_2 uptake, upon illumination, was similar to the steady-state rate. The activation of initial CO_2 uptake may not be as fast in this strain as in other cyanobacteria studied (e.g., *Synechococcus* sp. strain PCC 7942), leading to similar initial and gross steady-state rates.

DISCUSSION

As demonstrated previously (16), CO_2 uptake was abolished in the mutant (M55) of *Synechocystis* sp. strain PCC 6803 that lacked *ndhB*. CO_2 uptake was also inhibited in other *ndhB* mutants (B1 to B6 [Fig. 2]) that had been generated by random-site mutagenesis. However, in contrast to the previous observation with M55, inhibition of HCO_3^- uptake was not significant in these mutants and was at most 50%, even after complete inactivation of *ndhB* in M55 (Fig. 1 and 2). In other words, careful analysis of M55 indicates that inhibition of CO_2 uptake is the major effect and that inhibition of HCO_3^- is a minor effect. In the less disruptive B1 to B6 mutants, the difference is more specifically related to CO_2 uptake, indicating that the complete loss of the NdhB protein in M55, and potential loss of all NDH-1 complexes, may lead to secondary effects on HCO_3^- uptake. Moreover, mutants B1 and M55 grew as fast as the WT in liquid medium adjusted to pH 8.0 and gassed with air levels of CO_2 (Fig. 4), unlike the previous observation that M55 was unable to grow under these conditions (16). Since WT cells grew at pH 7.0 as fast as at pH 8.0, we did not pay much attention to the pH of the medium for growth of M55 in previous studies, and a medium of pH 7.0 was frequently used. The discrepancy between the present and previous observations possibly arose from a mistake in growing the M55 cells at pH 7.0. To test this possibility, we grew M55 at pH 7.0 under 3% CO_2 and then aerated with air overnight at the same pH. These M55 cells did not show HCO_3^- transport activity (Fig. 1), in contrast to the result obtained with the mutant cells grown and induced at pH 8.0 (Fig. 1 and 2). Thus, the slow growth or nongrowth of M55 under air reported in previous studies appears to be due to a failure to present the mutant with the more favorable conditions of pH 8.0 medium. In contrast, the B1 mutant grew almost as well as the WT at pHs 6.5, 7.0, and 8.0 under high-CO_2 conditions (Fig. 4).

The mutation of *ndhB* had much less effect on HCO_3^- uptake than on CO_2 uptake (Fig. 1 and 2), consistent with the observation that HCO_3^- transport appears to be driven by linear electron transport (8). The activity of HCO_3^- uptake in M55 was much lower than the WT rate (Fig. 1 and 2). This could be due to secondary effects of stress caused by exposing the cells to light under low-CO_2 conditions in the absence of CO_2 uptake. The possibility that NDH-1 has a secondary role in HCO_3^- transport in supplying additional ATP by an NDH-1-dependent cyclic electron transport around photosystem I cannot be ruled out (11–13). The CO_2 uptake reaction is postulated to be an energy-dependent unidirectional conversion of CO_2 to HCO_3^- (reviewed in reference 4). However, this reaction may be ATP independent, since CO_2 uptake is not operational in the presence of linear electron transport alone (8).

The inability of the *ndhB* mutants to grow at pH 7.0 or 6.5 under atmospheric levels of CO_2 correlated with their low capacities for CO_2 uptake (Fig. 2 and 3). Thus, the inorganic carbon source is mainly supplied by CO_2 uptake under these conditions. These mutants grew as fast as the WT in liquid medium at pH 8.0 under air, which indicated that at this pH, HCO_3^- transport was a sufficient carbon source for the cells. On agar plates, the mutant colonies grew more slowly than the WT at pH 8.0 under air.

The presence of five *ndhD* genes in the *Synechocystis* sp. strain PCC 6803 genome (3) and their differential expression under different CO_2 concentrations (21) suggested that there are multiple types of NDH-1 complexes with different functions. In this series of mutants, the *ndhD3* mutant was the only one that displayed the phenotype of slow growth at limiting CO_2 (i.e., 50 ppm CO_2) and reduced affinity for CO_2 uptake, whereas the other mutants lacking *ndhD* (*ndhD1*, *ndhD2*, *ndhD4*, and *ndhD5* mutants) did not show this mutant phenotype (Table 2 and Fig. 7). The $K_{0.5}$ value for CO_2 uptake was higher in the *ndhD3* mutant than in the WT (Fig. 7), which may explain the low growth rate of *ndhD3* at pH 6.5 and 50 ppm CO_2 (Table 2). Similar results have recently been reported with a mutant of *Synechococcus* sp. strain PCC 7002 in which the *ndhD3* gene was inactivated (5). None of the *ndhD* mutants showed the accentuated phenotype of M55 (i.e., complete loss of CO_2 uptake activity). Recently, we found that an *ndhD3-ndhD4* double mutant did not show any CO_2 uptake activity,

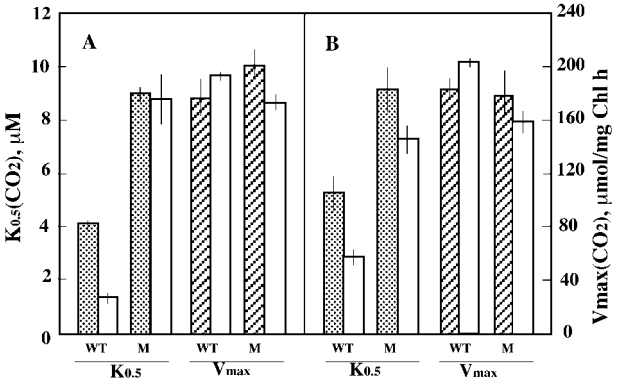

FIG. 7. The $K_{0.5}(CO_2)$ and V_{max} values for steady-state (A) and initial (B) CO_2 uptake by the WT and *ndhD3* mutant (M) strains of *Synechocystis* sp. strain PCC 6803 grown under 2% CO_2 (shaded and hatched bars) and after 18 h of induction under 20 ppm CO_2 (open bars). Each bar shows the average value of the results of four measurements, and the error bars indicate standard deviations.

although the mutant grew as fast as the WT under photoheterotrophic conditions at atmospheric levels of CO_2 (unpublished results). It appears evident that there are functionally distinct multiple NDH-1 complexes and that an NDH-1 complex having *ndhD3* or *ndhD4* as a subunit is essential to CO_2 uptake. The exact function of this NDH-1 complex in CO_2 uptake is not known and is being explored.

The information presented in this paper, however, does allow for limited speculation on how a particular type of NDH-1 complex might be specifically involved in CO_2 uptake but not HCO_3^- uptake. One previous model for CO_2 uptake in cyanobacteria suggested that a vectorial carbonic-anhydrase-like moiety located within a notional plasma membrane-based transporter could function in the transport of CO_2 by providing a localized source of hydroxyl ions to drive the conversion of CO_2 to HCO_3^- followed by release of HCO_3^- on the cytosolic face of the transporter (23). In this model, the CO_2 transporter would operate as a type of active, facilitated diffusion and the necessary OH^- ions were envisaged to be supplied by some component of the respiratory electron transport chain. A new speculative model has arisen (4) that postulates that a type of facilitated diffusion could operate at the level of the thylakoid and result in active accumulation of HCO_3^-. Here, a vectorial carbonic-anhydrase-like reaction could be closely associated with an NDH-1 complex such that OH^- ions might be produced in a "localized pocket" and used to drive the conversion of CO_2 to HCO_3^-. In this model, initial entry of CO_2 into the cell would be passive, whereas direct HCO_3^- uptake would occur via separate transporters, such as the *cmp* transporter, BCT1 (22). The results of this paper are consistent with this new model in that elimination or mutation of all potential NDH-1 complexes (i.e., *ndhB* modification) or modification of specific subcomplexes containing NdhD3 or NdhD4 result in partial or complete loss of CO_2 uptake capacity without major effects on HCO_3^- uptake. The recent finding that the NdhB protein is missing from highly purified cytoplasmic membranes of WT cells (results not shown) but is present in thylakoid preparations (Fig. 5) is also consistent with this new thylakoid-based model for CO_2 uptake.

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