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Functionally distinct NAD(P)H dehydrogenases and their membrane localization in *Synechocystis* sp. PCC6803

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Abstract. The type I NAD(P)H dehydrogenase complex (NDH-1) in cyanobacteria is involved in both respiratory and photosynthetic electron transport processes. NDH-1 is also essential for inorganic carbon transport. It has been postulated that NDH-1-dependent cyclic electron flow around PSI energizes CO₂ uptake. The genome information of *Synechocystis* sp. PCC6803 has enabled us to provide an integrative view of the CO₂ concentrating mechanism in this organism. In an attempt to dissect the role of the NDH-1 complex, we have constructed single and double mutants of *Synechocystis* 6803 by disrupting highly homologous *ndhD* genes in pairs, and have analysed the growth, CO₂ uptake activities, and redox levels of P700 and the plastoquinone pool in these mutants under various conditions. We have also determined the membrane localization of this membrane protein. Our studies have revealed that: (i) mutations in *ndh* genes lead to inhibition of CO₂ uptake, rather than HCO₃⁻ uptake; (ii) NDH-1 complexes are localized only in the thylakoid membrane; (iii) there are functionally distinct NDH-1 complexes in *Synechocystis* 6803. Based on these data, we propose a schematic view of the roles of different NDH-1 complexes in cyanobacteria.

Keywords: CO₂ uptake, cyanobacterium, cytoplasmic membrane, NAD(P)H dehydrogenase, *ndh*, *Synechocystis* 6803, thylakoid membrane.

Introduction

During the past 15 years, mutants affected in several distinct CO₂ concentrating mechanisms (CCMs) have been studied by many cyanobacteriologists. Kaplan and his colleagues first reported the isolation of high-CO₂ requiring (HCR) mutants defective in the CCM (Marcus *et al.* 1986). After this pioneering work, many mutants defective in the CCM have been isolated and analysed, using various transformable strains of cyanobacteria. Physical and molecular analyses of these mutants revealed that the cyanobacterial CCM consists of three basic systems: (i) inorganic carbon (Ci) transporters; (ii) a system for energizing Ci transport; (iii) the Rubisco-containing carboxysome system for CO₂ fixation. RKA and RKb are the HCR mutants of *Synechocystis* sp. PCC6803 that lack the ability to transport extracellular Ci into cells (Ogawa 1990). These mutants are impaired in the *ndhB* and *ndhL* genes, respectively. Both these genes encode subunits of the NDH-1 complex in the system (ii) described above

(Ogawa 1991a, b). These were the first reports suggesting that one of the CCM mutants resulted in defective NDH-1.

NDH-1 is essential for Ci transport in cyanobacteria. Using an *ndhB*-inactivated mutant of *Synechocystis* 6803 (M55), this enzyme was also shown to be a component of PSI cyclic electron flow (Mi *et al.* 1992, 1994, 1995). It has been assumed that ATP produced by PSI cyclic electron flow is the direct energy source for Ci transport (Ogawa 1991a, 1992). To understand the role of NDH-1 in Ci transport, we have investigated whether mutation of *ndhB* affects the uptake of CO₂ and HCO₃⁻.

The genome of *Synechocystis* 6803 contains genes for 12 subunits of NDH-1. The *ndhD* and *ndhF* genes are present as gene families with six and three members, respectively, although most other *ndh* genes are present as single copies. This suggests that several types of NDH-1 complex exist in cyanobacterial cells, each with different NdhD and/or NdhF subunits, and with each potential

Abbreviations used: CA, carbonic anhydrase; CCM, CO₂ concentrating mechanism; Ci, inorganic carbon; HCR, high-CO₂ requiring; NDH-1, NAD(P)H dehydrogenase complex; PQ, plastoquinone; ΔμH⁺, proton gradient.

complex having different function(s). To determine whether there are functionally distinct NDH-1 complexes, we have constructed double mutants of *Synechocystis* 6803 by disrupting highly homologous *ndhD* genes in pairs, and have analysed their growth under various conditions, CO₂ uptake activities, and redox levels of P700 and the plastoquinone (PQ) pool.

Membrane localization of the NDH-1 complexes is important to understand their functions. Toward this goal, we have performed immunoblot analysis of purified cytoplasmic and thylakoid membranes from *Synechocystis* 6803 cells.

Energy sources for HCO₃⁻ and CO₂ transport

In early studies of the energy source for Ci transport, Ogawa and coworkers (1983, 1985) used monochromatic light to demonstrate that the Ci uptake process is driven by PSI activity. Subsequently, studies of various mutants with mutation in genes encoding several subunits of the NDH-1 complex have demonstrated that NDH-1 is essential for Ci transport in cyanobacteria (Ogawa 1991*a, b*, 1992; Marco *et al.* 1993; Kaplan and Reinhold 1999). Mutations of *ndhB*, *ndhL* or *ndhK* in *Synechocystis* 6803 significantly reduced the activities of CO₂ and HCO₃⁻ uptake. The mutants of *ndhB* also lacked PSI dependent cyclic electron flow. Therefore, it has been assumed that the NDH-1-dependent cyclic electron transport supplies ATP to drive Ci uptake (Ogawa 1991*a, b*, 1992). The recent studies of the *cmp* operon (Omata *et al.* 1990), encoding components of an ATP binding cassette-type transporter, under various conditions, demonstrated that these genes encode an ATP-dependent HCO₃⁻ transporter (Omata *et al.* 1999). One could assume that the ATP produced by cyclic electron flow via NDH-1 is directly used in HCO₃⁻ transport processes. However, we do not know why ATP produced by linear electron transport in these mutants does not drive Ci transport. It is also not certain whether ATP energizes the uptake of CO₂ and HCO₃⁻. Based on the observation of differential effects of electron transport inhibitors and acceptors on the uptake of these two carbon species, Li and Calvin (1998) reported that HCO₃⁻ uptake is supported by linear electron transport, while CO₂ uptake is supported by cyclic electron transport. The results suggested that ATP produced by non-cyclic electron transport energized HCO₃⁻ transport. However, little is known about the mechanism of CO₂ uptake and how it is energized.

To help understand the role of NDH-1 in Ci transport, we investigated whether mutation of *ndhB* affects the uptake of CO₂ and HCO₃⁻ equally, and constructed various mutants by introducing mutations in the *ndhB* gene of *Synechocystis* 6803 by random mutagenesis. Figure 1 shows the relative amounts of CO₂ (left column) and HCO₃⁻ (right column) taken up by wild type, B1 (multiple point mutations in *ndhB*), and M55 (mutant lacking *ndhB*) cells grown at pH

8.0 under 3% CO₂, and then aerated for 18 h in the light. The activity of CO₂ uptake was very low in the B1 and M55 mutants, whereas the activity of HCO₃⁻ uptake in B1 was as high as that in wild type, and M55 still possessed half the wild type activity. We also analysed other mutants generated by random mutagenesis and obtained the same effect in these mutants (Ohkawa *et al.* 2000*a*). Thus, measurement of CO₂ and HCO₃⁻ uptake in these mutants clearly showed that CO₂ uptake is more strongly inhibited than HCO₃⁻ uptake (Ohkawa *et al.* 2000*a*). However, the result that HCO₃⁻ uptake was inhibited in the mutants indicates that NDH-1 has a supplemental role in the transport of HCO₃⁻. The secondary effect of stress, by exposing the cells to low CO₂ conditions, might account for this result.

Although we clarified the involvement of NDH-1 in uptake of CO₂ rather than HCO₃⁻, it is not certain how CO₂ is transported into the cells. Active transport of CO₂ across the cytoplasmic membrane has been proposed (Espie *et al.* 1991; Miller *et al.* 1991). However, Tchernov *et al.* (2001) recently reported that CO₂ uptake was greatly inhibited by a water channel blocker. They have suggested that the transport of CO₂ across the cytoplasmic membrane occurs passively via a water channel. Based on their recent findings, it is likely that the energy for CO₂ uptake is produced on the thylakoid membrane where NDH-1 is located.

Localization of NAD(P)H dehydrogenase

Identification and localization of proteins is essential to elucidation of their functions. The $\Delta ndhD3$ mutant in *Synechococcus* sp. PCC 7002 did not show an effect on cyclic electron flow (Klughhammer *et al.* 1999), and the

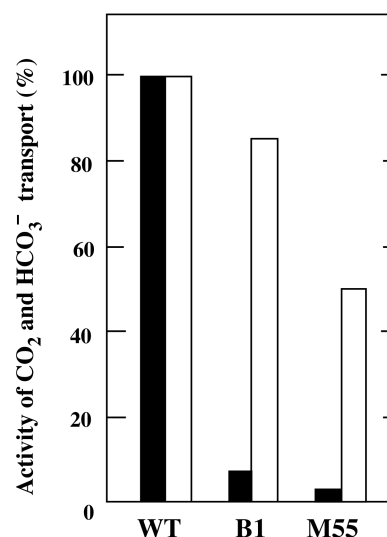


Fig. 1. ¹⁴CO₂ (filled columns) and H¹⁴CO₃⁻ (open columns) transport activities in wild type (WT), B1 (multiple point mutations in *ndhB*), and M55 cells. Each value is shown as a percentage of the value obtained for WT cells.

authors of this article suggest that the NDH-1 containing this subunit is located on the thylakoid membrane. It has been postulated that NDH-1 is present both in the thylakoid and cytoplasmic membranes, basically because antibodies raised against NdhJ and NdhK detected these proteins in both types of membranes from *Synechocystis* 6803 (Berger *et al.* 1991; Pieulle *et al.* 2000). However, questions have been raised about the purity of the membrane preparations used in these studies (Ogawa 1992; Norling *et al.* 1998). Norling *et al.* (1998) have recently developed a new method using aqueous polymer two-phase partitioning, in combination with sucrose density gradient centrifugation, to isolate highly purified cytoplasmic and thylakoid membranes from *Synechocystis* 6803. We isolated highly purified thylakoid and cytoplasmic membranes using this new method and attempted to determine the cellular location of NDH-1.

At first, we tried to check for cross-contamination of the two types of membranes in various fractions after sucrose density gradient centrifugation using antibodies raised against NrtA and CP43, two proteins used as markers for the cytoplasmic and thylakoid membranes, respectively. The panels *A* and *B* in Fig. 2 show immunodetection of NrtA and CP43 in highly purified cytoplasmic and thylakoid membranes, respectively, establishing the purities of the isolated thylakoid and cytoplasmic membrane preparations. These results are consistent with those shown by Norling *et al.* (1998). The antibodies against NdhH and NdhB recognized these proteins only in the thylakoid membrane fractions, and not in the cytoplasmic membrane fractions (panels *C* and *D* in Fig. 2). These results demonstrated that the NDH-1 complex is localized in the thylakoid membrane. Evidently, the specific site of reactions involving NDH-1, such as electron transport, proton translocation, and active conversion of CO₂ to HCO₃⁻ (leading to net CO₂ uptake), is in the thylakoid membrane.

Functionally distinct multiple NAD(P)H dehydrogenases

An analysis of the genomic sequence of *Synechocystis* 6803 (Kaneko *et al.* 1996; <http://www.kazusa.or.jp/cyano>) shows the presence of 12 subunits of NDH-1 complex in this organism. This complex contains large hydrophobic subunits. For example, NDHB, NDHD, and NDHF are integral membrane components (Weidner *et al.* 1993). It is clear that most *ndh* genes are present as single copies in *Synechocystis* 6803. However, *ndhD* and *ndhF* are present as multiple copies with six and three members, respectively, suggesting that several types of NDH-1 might exist in this cyanobacterium, each with different functions of the NdhD and NdhF subunits (Ohkawa *et al.* 1998; Price *et al.* 1998; Klughammer *et al.* 1999). Some *ndh* mutants, such as the M55 mutant (*ndhB*), show complete inhibition of CO₂ uptake (Ogawa 1991; Ohkawa *et al.* 2000a), whereas other mutants, such as *ndhF1* or *ndhD3* (K22 and A41 in *Synechococcus*), show no effect on Ci transport, or show

effects only on high affinity CO₂ uptake (Yu *et al.* 1993; Klughammer *et al.* 1999). The *ΔndhD3* mutant of *Synechocystis* 6803 grows poorly under limiting CO₂ conditions (i.e. 50 ppm CO₂), and exhibits reduced affinity for CO₂ uptake. However, *ndhD*-less mutants (*ΔndhD1*, *ΔndhD2*, *ΔndhD4* and *ΔndhD5*) lack such a phenotype, suggesting that there are functionally distinct NDH-1 complexes in cyanobacteria.

To test this hypothesis, we focused on the gene families, such as *ndhD* and *ndhF*, and tried to provide a consensus view of the function of NDH-1 in cyanobacteria. We compared the amino acid sequences and identified three groups. We then constructed double mutants of *Synechocystis* 6803 by disrupting highly homologous *ndhD* genes in pairs, and analysed their growth, CO₂ uptake, and redox levels of P700 and the PQ pool, under various conditions.

The ability of cells to grow under photoheterotrophic conditions strongly depends on their respiratory activity. The *ΔndhD1/D2* and M55 mutants, that exhibited low respiration rates, were unable to grow under photoheterotrophic conditions (Fig. 4). Reduction of P700 was also strongly inhibited in these mutants (Fig. 4). This indicated that the NdhD1/NdhD2 type of NDH-1 complex principally mediate the transport of electrons from NADPH to the PQ pool. The single *ΔndhD3* mutant exhibited CO₂

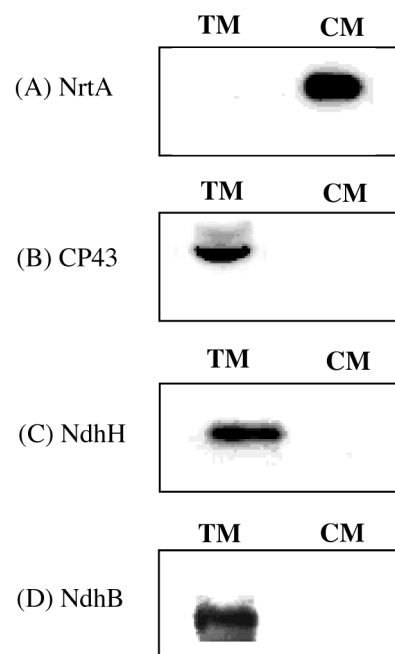


Fig. 2. Immunodetection of NrtA (*A*), CP43 (*B*), NdhH (*C*), and NdhB (*D*) in highly purified thylakoid (TM) and cytoplasmic (CM) membranes obtained after sucrose gradient centrifugation. Each lane was loaded with membranes containing 15 μg protein. NrtA is a subunit of an ABC-type nitrate transporter, and CP43 is a chlorophyll-binding protein component of PSII.

uptake activity less than that of wild type cells (Fig. 3). Moreover, the $\Delta ndhD3/D4$ double mutant was unable to take up CO_2 , a phenotype similar to that of the M55 mutant (Ohkawa *et al.* 2000b). It is evident that only the NdhD3/NdhD4 type of NDH-1 complex is essential for CO_2 uptake. Thus, it appears that there are two types of functionally distinct NDH-1 complex in *Synechocystis* 6803, and M55 exhibits the phenotype of both types of the mutants (Fig. 5). The $\Delta\mu\text{H}^+$ generated by photosynthetic electron flow is used as the direct source of energy for uptake of CO_2 (Tchernov *et al.* 2001). It is apparent that NDH-1 is essential for CO_2 uptake on the thylakoid membrane (Fig. 5, and see Ohkawa *et al.* 2001). Thus, the driving force for inward diffusion of CO_2 is provided by the conversion of CO_2 to HCO_3^- at the thylakoid membrane.

It has been assumed that PSI cyclic electron flow is essential for CO_2 uptake in cyanobacteria (Ogawa *et al.* 1985; Mi *et al.* 1992, 1994, 1995; Li and Calvin 1998). The NdhD1/NdhD2 type of NDH-1 is essential for the PSI cyclic electron flow, but is not needed for CO_2 uptake. The NdhD3/NdhD4 type of NDH-1 is essential for CO_2 uptake. These results indicate that PSI cyclic electron flow is not needed for CO_2 uptake. Thus, the previous hypothetical model appears to be unlikely. To reconcile the former suggestions, we have proposed a scheme for different routes of cyclic electron flow (Fig. 5). The results of P700 oxidation show the existence of different routes for electron flow, depending on the different NDH-1 complexes (Figs 4, 5). In recent work, we have distinguished these two

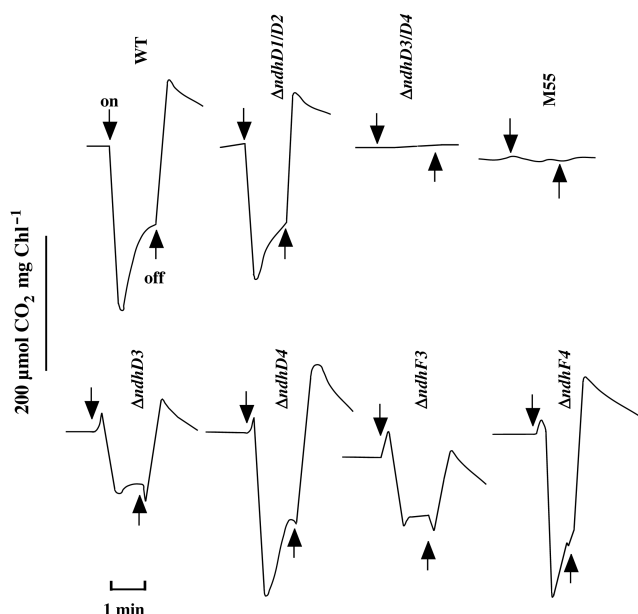


Fig. 3. CO_2 exchange in wild type (WT) and mutant cells grown under air conditions. The rate of CO_2 exchange was recorded as a function of time using an open infrared gas analysis system (Ogawa *et al.* 1985). \uparrow , light on; \downarrow , light off.

NDH-1-dependent CO_2 -uptake systems based on the kinetics of CO_2 uptake (Shibata *et al.* 2001). The NDH-1B1 associated with NdhD3, and the NDH-1B2 associated with NdhD4, involved in inducible and constitutive CO_2 uptake systems, respectively, are essential for CO_2 uptake but not for PSI cyclic electron flow (Fig. 5). These observations raise the possibility of alternate electron acceptors for different functional NDH-1 complexes (Fig. 5 and see Jeanjean *et al.* 1998; Tchernov *et al.* 2001). The distribution of PSI and PSII complexes in another unicellular cyanobacterium, *Synechococcus* PCC7942 is heterogeneous

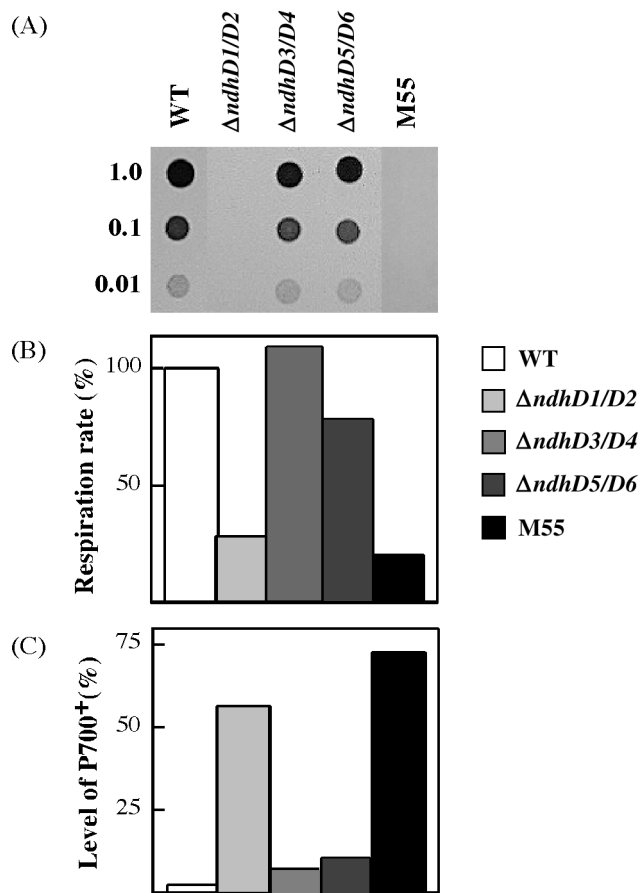


Fig. 4. (A) Growth of wild type (WT) and mutant cells under photoheterotrophic conditions. Cell suspensions (2 μL) with the OD (730 nm) of 1.0 (upper row), 0.1 (middle row), and 0.01 (lower row) were spotted. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU; 10 μM) was added to the plates for photoheterotrophic growth under an air level of CO_2 . (B) Respiration rates of WT and mutant cells. Oxygen consumption by intact cells was measured in darkness on a Clark-type oxygen electrode (Ohkawa *et al.* 2000a). Each value is shown as a percentage of the value obtained for WT cells. (C) Oxidation level of P700 in WT and mutant cells. The P700⁺ levels were normalized assuming that P700 was completely oxidized when a 50 μs pulse of saturating white light (150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) was applied to the cell suspension. Each value shows the levels of P700⁺ at 3.3 $\mu\text{eq. m}^{-2} \text{s}^{-1}$ of far-red light.

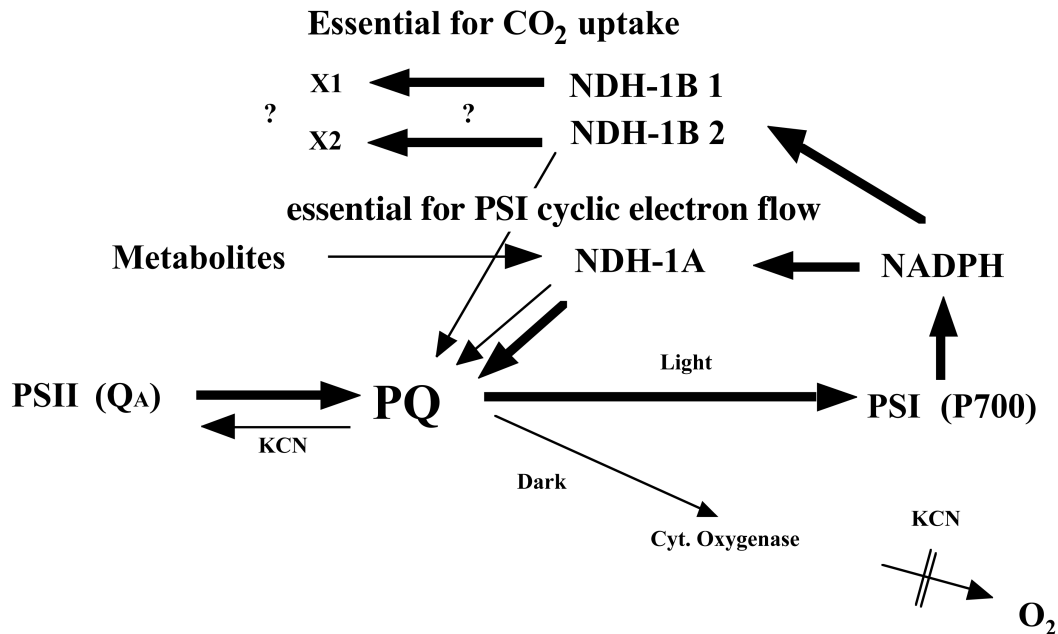


Fig. 5. A hypothetical model of the roles of NDH-1 complexes in energetics, and in the inducible and constitutive CO₂ uptake systems, of cyanobacteria. This figure shows the electron transport pathways in which the two functionally distinct NDH-1 complexes, NDH-1A (NdhD1/NdhD2 type) and NDH-1B (NdhD3/NdhD4 type), participate. NDH-1A is essential for cyclic electron flow around PSI. The NDH-1B1 (associated with NdhD3) and NDH-1B2 (associated with NdhD4) complexes are involved in inducible and constitutive CO₂ uptake systems, respectively. They are essential for CO₂ uptake, but not for PSI cyclic electron flow. X1 and X2 are unknown electron acceptors. PQ, plastoquinone; Q_A, tightly bound plastoquinone.

(Sherman *et al.* 1994), raising the possibility that the PSI population in cyanobacteria might be functionally heterogeneous. This may indicate that different routes for cyclic electron flow are functional due to multiple PSI types. Intracellular conversion of the entering CO₂ to HCO₃⁻ is mediated by carbonic anhydrase (CA) activity (Volokita *et al.* 1984; Abe *et al.* 1987; Price and Badger 1989; Kaplan and Reinhold 1999). But the CA-like moiety has not been identified.

The role of NDH-1 complexes, especially in CO₂ uptake mechanism, is poorly understood. Ogawa and coworkers (T. Ogawa, unpublished data) have transformed a transposon-bearing library of *Synechocystis* 6803 DNA into the *ndhD3* mutant, and have isolated several mutants that are unable to grow at pH 7.0 in air. It is hoped that the analysis of some of these mutants will greatly help us to further understand Ci transport, particularly associated with NDH-1, in cyanobacteria.

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