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The gene encoding the NdhH subunit of type 1 NAD(P)H dehydrogenase is essential to survival of *Synechocystis* PCC6803

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Abstract The physiological function of the type 1 NAD(P)H dehydrogenase (Ndh-1) of *Synechocystis* sp. PCC6803 has been investigated by inactivating the gene *ndhH* encoding a subunit of the complex. Molecular analysis of independent transformants revealed that all clones were heteroploid, containing both wild-type and mutant *ndhH* copies, whatever the metabolic conditions used during genome segregation, including high CO_2 concentration. By replacing the chromosomal copy of the *ndhH* gene by a plasmidial copy under the control of a temperature-controlled promoter, we induce a conditional phenotype, growth being only possible at high temperature. This clearly shows for the first time that an *ndh* gene is indispensable to the survival of *Synechocystis* sp. PCC6803.

Key words: Cyanobacteria; *Synechocystis* PCC6803; NAD(P)H dehydrogenase; *ndh* gene; Temperature-controlled expression

1. Introduction

Cyanobacteria are the only cells capable of performing both aerobic respiration and oxygenic photosynthesis in the same cellular compartment. Besides carriers involved in photosynthetic electron transport, thylakoid membranes have been shown to contain respiratory electron transport carriers such as a type 1 NAD(P)H dehydrogenase (Ndh-1 complex) and a terminal oxidase (see [1] for a review). The Ndh-1 complex catalyzes an electron transfer from NADH or NADPH to quinones, linked to the translocation of protons across the membrane. Genes encoding 11 of the 14 subunits of the minimal complex I have been found in the genome of the cyanobacteria *Synechocystis* sp. strain PCC6803 [2]. Attempts to isolate and purify an active cyanobacterial Ndh-1 complex have been unsuccessful but a subcomplex containing different *ndh* gene products (NdhK, NdhJ, NdhI and NdhH) has been purified from *Synechocystis* PCC6803 [3]. Based on immunological data, it was concluded that Ndh subunits are located both in plasma and thylakoid membranes, thus suggesting that a functional Ndh-1 complex is present in both membranes [4].

The physiological function of the Ndh-1 complex is not yet fully understood probably due to the existence of conflicting data regarding the phenotypical analysis of *ndh* gene disrupted mutants. In most *ndh* disrupted mutants, respiration has been shown to be impaired, thus indicating that *ndh* gene products and most probably the Ndh-1 complex are involved in a respiratory electron transport chain. Inactivation of some *ndh* genes (*ndhB*, *ndhK* or *ndhL* genes) [5–7] resulted in a reduced ability of mutant strains to accumulate C_i. It was initially proposed that cyclic electron flow around photosystem I (PSI) mediated by the Ndh-1 complex, may be involved in the production of energy required for HCO_3^- transport [5]. Recent investigations performed on several *ndhB* mutants concluded that CO_2 uptake, rather than HCO_3^-, was impaired in these mutants [8]. In *Synechocystis* genome, some *ndh* genes are present in single copy number (*ndhB*, *ndhH*, *ndhK*, ...), while others (*ndhD* and *ndhF*) are present in multiple copy number (the cyanobase, [2]). Interestingly, inactivation of the different copies of multiple copy genes led to contrasted phenotypes. Among the different *ndhD* mutants, the *ndhD3* mutant was the only mutant to display a phenotype of slow growth at limiting CO_2 [8,9]. A similar phenotype was reported for the *ndhF3* deleted mutant [8]. Similar data were obtained in *Synechococcus* PCC7002 [10]. Based on the differential expression of multicopy *ndh* genes and on the existence of contrasted phenotypes of inactivated mutants, the existence of different Ndh-1 complexes with different functionalities and subunit compositions was proposed [8–12]. In other cyanobacterial species, like *Plectonema boryanum* or *Anabaena* PCC7120, attempts to inactivate *ndhA*, *I*, *G* or *E* and *ndhK*, respectively, have been unsuccessful, thus suggesting that these genes are essential in these cyanobacterial species (Y. Takahashi, unpublished data, [13]). However, until now, no reports have concluded that any of the *ndh* genes is essential to the survival of *Synechocystis* PCC6803.

In an attempt to further study the role of *ndh* genes in *Synechocystis* PCC6803, we decided to inactivate the *ndhH* gene present in single copy in the genome. By using a temperature-controlled expression vector [14], we clearly demonstrate

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Abbreviations: Km, kanamycin; Cm, chloramphenicol; Sm, streptomycin; Sp, spectinomycin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

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for the first time, and in contrast to another single copy *ndh* gene (*ndhB*), that the *ndhH* gene is essential to the viability of *Synechocystis* PCC6803 whatever the CO_2 concentration supplied in the culture medium. We propose, in agreement with recent studies, the existence of different Ndh complexes characterized by different functionalities and subunit compositions.

2. Materials and methods

2.1. Strains, growth conditions and gene transfer procedures

Escherichia coli strains were grown on Luria–Bertani (LB) medium at 37°C (HB101) or at 30°C (CM404) and were used as hosts for gene manipulation (HB101) or conjugative plasmid transfer (CM404) to *Synechocystis* sp. strain PCC6803 [14]. *Synechocystis* was transformed as described [15] and subsequently grown on BG11 medium [16] at temperatures ranging from 30 to 38°C under various intensities of white light: standard light (SL = 30 μE m^{-2} s^{-1}) or low light (LL = 10 μE m^{-2} s^{-1}) in the presence or absence of 55 mM glucose. Modified Allen's mineral medium [17] was also used for growth under high inorganic carbon concentration (12 mM HCO_3^-) in plates. Additionally, high CO_2 concentration in air was produced by placing Petri dishes in plexiglass cabinets flushed with moistened 2% CO_2 enriched in air. The high CO_2 requiring mutant M55 (*ndhB*::kanamycin (Km)^r) of *Synechocystis* PCC6803 (kindly provided by Dr. T. Ogawa) was routinely cultivated in modified Allen's mineral medium.

2.2. Nucleic acid manipulations

Synechocystis RNA was extracted and hybridized [18] to specific DNA probes synthesized by polymerase chain reaction (PCR) and labelled with digoxigenin (Boehringer Mannheim). For Northern blot experiments, even loading of the RNA samples was controlled by A_{260} readings and by verification of equal abundance of the rRNA bands on the agarose gel. Transcript sizes were estimated from the migration rates on agarose gels using various standards: rRNA from *Synechocystis* and RNA molecular weight marker from Boehringer (0.3–7.4 kb) and from Gibco BRL (0.24–9.5 kb).

2.3. Construction of the plasmid for targeted deletion of the ndhH gene

The chloramphenicol (Cm)^r cassette used for *ndhH* inactivation (pΔndhH plasmid) was constructed as follows. First, the DNA region upstream of the *ndhH* coding sequence was amplified by PCR using the *Nar*I creating primer (5′-TAATCAATTCTTCCCCGGCCATGG-GCGCCATTACTTCGG-3′) and the *Bam*HI creating primer (5′-AT-AGACGGGTGATGGATCCCCATGTTGAGCAC-3′). This PCR product (F_A) was cleaved with both *Nar*I and *Bam*HI and was cloned in the Ap^{r}Cm^r vector, pPMB12B opened with the same enzymes, yielding the p$\Delta H/F_A$. Then, the chromosomal region encompassing the nucleotides 725–1185 of the *ndhH* coding sequence and the downstream DNA region was also amplified by PCR using two *Hin*dIII creating primers (5′-GCTACGACAAGCTTGATTGGGAAG-3′ and 5′-GAGCGACGCTAAGCTTTAACGACCTCATTAGCCATGGG-3′). This second PCR fragment was cloned in p$\Delta H/F_A$ after *Hin*dIII restrictions. The plasmid obtained, pΔndhH harbors the *cat* gene between *ndhH* flanking chromosomal DNA regions, which served as platforms of homology for recombinations mediating gene replacement in *Synechocystis*. The deletion cassette of pΔndhH plasmid was sequenced before and after transformation to *Synechocystis*. The following primers, $sll252_5$′ (5′-CCGCTATTAGTTTTGCCA-TTGGCG-3′) and $slr262_3$′ (5′-CCGTCTCCGTCTCCACTTCAGA-CT-3′) were used for testing by the PCR method the segregation of *Synechocystis ndhH* modified strains (Fig. 2C).

2.4. Construction of the replicative vector for conditional expression of ndhH

The Cm^{r}streptomycin (Sm)^r/spectinomycin (Sp)^r RSF1010-derived plasmid pFC1 that replicates autonomously in several organisms [14] was used for temperature-controlled expression of the wild-type *ndhH* gene of *Synechocystis*. The *ndhH* coding region was amplified by PCR using the *Nde*I creating primer (5′-GATAGCAGTTAACCGCATAT-GACCAAGATTGAAACCAG-3′) to introduce an *Nde*I restriction site in front of the ATG start codon, and the *Eco*RI creating primer (5′-GAACTGGGCAACGTCGAATTCCTAGCGGTCCACCGATC-CC-3′) to generate an *Eco*RI site downstream of the TAA stop codon. This PCR product was restricted with both *Nde*I and *Eco*RI and was cloned in pFC1 opened with the same enzymes, yielding the Cm^{s}Sm^r/Sp^r plasmid pFCndhH$_{6803}$. The plasmid was introduced by conjugation in the heteroploid strain of *Synechocystis*, and it's passenger DNA insert was sequenced before and after propagation in cells.

2.5. Electrophoresis and immunostaining

Cytoplasmic and thylakoid membranes were prepared from wild-type and M55 cells as described [19]. Typically, less than 0.015 μg of chlorophyll (Chl) per μg of membrane protein was present in wild-type and mutant cytoplasmic membrane preparations. In prepared thylakoid membranes, 0.25 μg of Chl per μg of membrane protein was present. Protein content was determined using a modified Lowry method, using bovine serum albumin as a standard (Sigma kit ref. B5656). Thylakoid and cytoplasmic proteins were separated by SDS–PAGE according to Laemmli [20]. Proteins were subsequently electrotransferred to nitrocellulose and reacted with the antibodies. Bound antibodies were detected using anti-rabbit Ig-G conjugated to alkaline phosphatase (Boehringer). The rabbit antisera raised against *Synechocystis* PCC 6803 NdhK and NdhI proteins that were used in this study were kindly provided by Dr. K. Steinmüller (University of Heinrich-Heine, Düsseldorf, Germany). The rabbit antisera raised against *Nicotiana tabacum* NdhH protein used in this study were described previously [21].

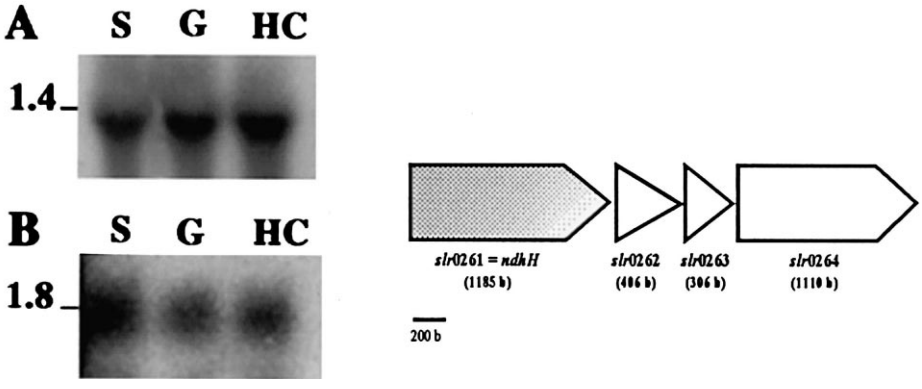

Fig. 1. Northern blot analysis of the *ndhH* gene from *Synechocystis*. Total RNA were isolated from cells grown on plates under the following conditions: standard (S), with glucose (G), with $NaHCO_3$ (high for inorganic carbon concentration, HC), electrophoresed on agarose gel and hybridized to probes covering the nucleotides 5–1184 of the *ndhH* reading frame (*slr*0261, A) or the nucleotides 3–391 of the *slr*0262 reading frame (B). Transcript sizes (in kb) are indicated at the side. Genetic organization around the *Synechocystis ndhH* gene was indicated beside the blots.

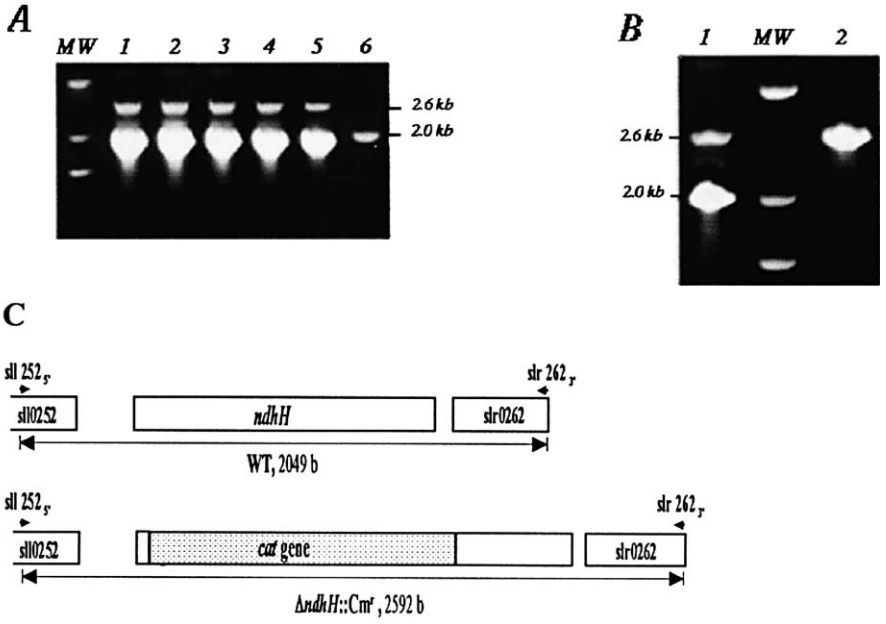

Fig. 2. A: PCR segregation analysis of a typical Cm^r transformant clone grown under one of the following conditions: standard (1), low light (10 μE m^{-2} s^{-1}) (2), high CO_2 (2% CO_2 in air) (3), high CO_2 (modified Allen's mineral medium) (4), high CO_2 (2% CO_2 in air plus modified Allen's mineral medium) (5). Lane 6, wild-type control cells grown under photoautotrophic standard conditions. Molecular weight marker (MW): 1636, 2036 and 3054 bases. B: Influence on chromosome segregation in *Synechocystis* of the temperature-controlled expression of the *ndhH* gene propagated on a replicating plasmid, $pFCndhH_{6803}$: PCR segregation analysis using oligonucleotide primers ($sll252_{5'}$/$slr262_{3'}$) of a typical Cm^rSm^r conjugant, lanes 1 (30°C) and 2 (38°C). Molecular weight marker (MW): 1636, 2036 and 3054 bases. C: Theoretical sizes of the DNA fragments amplified by PCR using oligonucleotide primers ($sll252_{5'}$/$slr262_{3'}$) complementary to the DNA regions flanking the *ndhH* gene are indicated on schematic maps of *Synechocystis* chromosome copies with (wild-type) or without (Δ*ndhH*::Cm^r) the *ndhH* gene.

3. Results

3.1. Inactivation of ndhH revealed that NdhH protein is required for cell viability in Synechocystis

We first attempted to delete the *ndhH* gene from all copies of the polyploid chromosome, i.e. about 10 per cell in *Synechocystis* [22]. The total sequence of the *Synechocystis* genome has revealed three putative genes (slr0262, slr0263 and slr0264) closely located upstream of the *ndhH* gene (slr0261) (Fig. 1). Before proceeding to *ndhH* inactivation we checked that these putative genes are expressed independently of the *ndhH* gene. In agreement with Steinmüller [23], *ndhH* was found to be expressed as monocistronic transcript of about 1.4 kb (Fig. 1A) while the upstream open reading frame (ORF) slr0262 seemed to be expressed as a polycistronic transcript (one labeled transcript of about 1.8 kb) with ORFs slr0263 and slr0264 (Fig. 1B). Fig. 1A also shows that no significant differences in the *ndhH* transcript levels are observed in different growth conditions, indicating that the *ndhH* gene is not transcriptionally controlled in response to the addition of glucose or $NaHCO_3$ to the medium.

Inactivation of the *ndhH* gene was carried out using a plasmid, pΔH (Section 2) containing the *ndhH* gene in which nucleotides 66–724 of the reading frame (1–1185) were replaced by the *cat* gene and where the *ndhH* flanking chromosomal regions cloned in the plasmid allow homologous recombination [22]. The 3′end of the *ndhH* coding region which may correspond to the slr0262, slr0263 and slr0264 promoter region was not modified thus avoiding possible polar effects of *ndhH* inactivation on the expression of downstream genes. pΔH was subsequently introduced in photoautotrophically grown *Synechocystis* cells by transformation. Successive streaking of Cm^r transformants onto plates were performed in the presence of increased Cm concentrations (from 5 to 25 μg ml^{-1}) to favor the amplification of mutant chromosome copies harboring the *cat* gene in place of *ndhH* gene. In addition to photoautotrophic growth conditions, various conditions of allele segregation were tested. Under mixotrophic conditions (addition of glucose), Cm^r colonies died during subsequent platings thus indicating that inactivation of the *ndhH* gene is lethal under these conditions. Since inactivation of some *ndh* genes (*ndhB*, *L*, *K*, [5–7]) has been previously reported to confer a high CO_2 requiring phenotype, we also streaked Cm^r colonies several times in the presence of high level of CO_2. Electrophoretic analysis of DNA fragments amplified by PCR in Cm^r colonies (more than 10 colonies in every case) showed that the Cm^r marker had been inserted properly in their genomes, thereby replacing the *ndhH* gene, but in only a minority of chromosome copies (hereafter designated as Δ*ndhH*::Cm^r), whereas the other copies remained wild-type irrespective of the conditions and the duration of subcultivation (more than 6 months in every case) (Fig. 2A). These results suggest that, in contrast to other Ndh subunits, the *ndhH* gene is essential to the survival of *Synechocystis* even under high CO_2 growth conditions.

In order to confirm this assumption, we constructed a strain completely lacking chromosomal *ndhH* alleles by using a temperature-controlled expression vector pFC1 [14]. The Sm^r/Sp^r plasmid $pFCndhH_{6803}$ (Section 2) was introduced by conjugation in the heteroploid *Synechocystis* strain ΔndhH::Cm^r/$ndhH^+$. Conjugants were grown either at 30°C (negative control) or at 38°C (heat induction) and DNA content was analyzed by PCR. Fig. 2B showed that heat-induced cells could segregate. In contrast, cells grown at 30°C remained hetero-

ploid. The segregated *ndhH* strain of *Synechocystis* (ΔndhH::Cm^r/pFCndhH$_{6803}$) is a conditionally lethal mutant. At 38°C under autotrophic, mixotrophic, heterotrophic (10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)+55 mM glucose) or high CO_2 growth conditions, this strain grows at the same rate as the wild-type thanks to *ndhH* expression from pFCndhH$_{6803}$. However, this strain does not survive at 30°C under the same conditions (Fig. 3). These observations clearly demonstrate that *ndhH* is essential for the survival of *Synechocystis* under the different growth conditions tested.

3.2. Immunological analysis of the ndhB-inactivated mutant (M55)

In order to explore why conflicting data have been obtained concerning inactivation of different *ndh* genes in *Synechocystis*, immunological analysis was performed on the *ndhB*-inactivated mutant (M55) strain to determine the presence or the absence of the Ndh subunits, NdhK, J and H. Fig. 4 shows that both cytoplasmic and thylakoid M55 membranes contain noticeable amounts of the NdhK subunit. Note that NdhJ and NdhH subunits were also detected in both types of membranes (data not shown) and that detected amounts of NdhK, J and H proteins were lower in the M55 mutant compared to wild-type (Fig. 4).

4. Discussion

To investigate the physiological function(s) of the Ndh-1 complex in *Synechocystis* PCC6803, we have attempted to delete the *ndhH* gene from the polyploid chromosome of this organism. We found that chromosome segregation was not complete (i.e. cells retained wild-type copies of the chromosome) irrespective of the growth conditions tested including high CO_2 which can compensate for the lack of various Ndh-subunits (NdhB, L, K and D3), [5–10,24]. This finding indicates that the *ndhH* gene is critical to the survival of cyanobacterial cells. This interpretation has been confirmed by using a temperature-controlled expression vector [14]. As expected, full chromosome segregation was achieved after heat-induction (38°C) of the plasmidial *ndhH* gene, and these cells died from the lack of NdhH when shifted back to the standard growth temperature (i.e. 30°C, where expression from the plasmid is repressed). Therefore, we clearly demonstrate for the first time, that an *ndh* gene is essential to the growth and survival of *Synechocystis* PCC6803. In the same species, Steinmüller et al. [25] isolated a strain in which *ndhK* inactivation was possible after activation of a cryptic homolog of *ndhK*, located on a large plasmid and it was concluded that *ndhK* is likely essential. In contrast, Ogawa [7] isolated a *Synechocystis* mutant, M-*ndhK*, unable to grow under CO_2 air levels or under photoheterotrophic conditions. Therefore, a second *ndhK* gene exists in this species, but its activation seems to depend on growth conditions prevailing during inactivation of the chromosomal *ndhK* gene. Nevertheless, the existence of a cryptic homolog of *ndhK* suggests that the NdhK product is important in *Synechocystis* as in *Anabaena* [13]. Some *ndh* genes have been previously reported to be essential to the growth of other cyanobacterial species. For instance, attempts to inactivate the *ndhA*, *ndhI*, *ndhG* or *ndhE* genes in the *P. boryanum* have been unsuccessful, suggesting an essential role in this organism (Y. Takahashi, unpublished data).

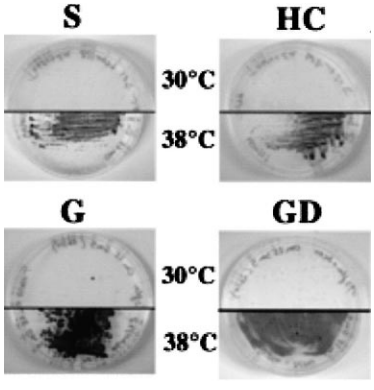

Fig. 3. Growth of the ΔndhH::Cm^r/pFCndhH$_{6803}$ strain at 38 and 30°C. Cells were grown at 38°C under standard conditions and then streaked on fresh plates and incubated at 38 and 30°C under one of the following conditions: standard (autotrophic, S), under modified Allen's mineral medium (high for inorganic carbon concentration, HC), with glucose (mixotrophic, G) and with glucose+DCMU, 10 μM (photoheterotrophic, GD).

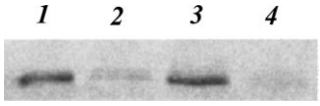

Fig. 4. Immunological analysis of thylakoid and cytoplasmic membranes from *Synechocystis* PCC 6803 (wild-type) and from an *ndhB*-disruption mutant (M55) using anti-NdhK antibodies. Thylakoid and cytoplasmic membranes were separated by SDS–PAGE electrophoresis and transferred to nitrocellulose membrane. Each lane contains 15 μg of proteins. Lane 1: wild-type thylakoid membranes, lane 2: M55 thylakoid membranes, lane 3: wild-type cytoplasmic membranes, lane 4: M55 cytoplasmic membranes.

The central question arising from our study is then: why inactivation of some different *ndh* genes is possible resulting in a high CO_2 requiring phenotype, whereas other *ndh* genes are indispensable for growth? One possible explanation would be to consider the existence of multiple forms of Ndh-1 complexes that would differ in composition and/or function as previously suggested by some authors [8–12]. In this context, and according to the present study, the NdhH subunit would be an essential subunit of all potential Ndh-1 complexes. Interestingly, among the amino acid sequences of the known Ndh proteins, the amino acid sequence of NdhH is the most conserved across large phylogenetic distances which is in agreement with a key role of the NdhH subunit in the Ndh-1 complexes in cyanobacteria [26]. It should be noted that NdhH is homologous to NuoD, one of the proteins belonging to the connecting fragment of the bacterial complex I. In line with this view, NuoD has been reported to play a central role in the *E. coli* NADH dehydrogenase [27] where it is involved in the structure of a 'quinone ring' subsite of complex I in *Rhodobacter capsulatus* [28]. The cyanobacterial NdhH subunit may therefore interact with plastoquinones, thus explaining a central and essential role of this subunit in Ndh-1 complexes. In contrast, NdhB would be involved in other forms of complexes participating in the cyclic electron flow around PSI. In agreement with this interpretation, immunological analysis performed in this study has shown that noticeable amounts of

three Ndh subunits (NdhH, J and K) are present in both thylakoid and cytoplasmic membranes of the *ndhB*-inactivated mutant M55. At this stage, it is not clearly demonstrated however that these three proteins are part of an active Ndh-1 complex in M55. In the case of *ndhD* and *ndhF* genes, the existence of five and four copies, respectively, of these genes in the genome of *Synechocystis* (the cyanobase, [2]) may reflect a participation of the different encoded subunits to particular forms of the complex. In line with this view, Ohkawa et al. [11] have demonstrated by Northern analysis that *ndhD1* and *ndhD4* are constitutively expressed, whereas *ndhD2*, *ndhD3* and *ndhD5* expression is induced by low CO_2. Moreover, only the mutant lacking *ndhD3* grew much more slowly than the wild-type under limiting CO_2 conditions [8,9]. In the same way, strong variations in the amounts of *ndh* transcripts in *Synechocystis* have suggested the existence of different forms of the enzyme under different growth conditions [26]. In *Synechococcus* sp. PCC7002, NdhD3 and NdhF3 subunits are only involved in high affinity CO_2 uptake [10], whereas inactivation of *ndhB* in *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC7942 leads also to a severe decline in cyclic flow and respiration in addition to Ci uptake [5,24].

Despite strong homologies between cyanobacterial and chloroplast *ndh* genes, inactivation of *ndh* genes leads to rather different phenotypes in cyanobacteria and in higher plants. In *N. tabacum*, different inactivation studies have concluded that *ndh* genes are dispensable for growth, although the existence of a functional Ndh complex, disappearing in response to *ndh* gene disruption, could be revealed [21,29,30]. Burrows et al. [29] reported that the NdhI subunit is undetectable from thylakoid membranes isolated from tobacco mutants in which *ndhC*, *ndhK* and *ndhJ* plastid genes had been disrupted. Also, the NdhH subunit could not be detected in a tobacco mutant in which the *ndhB* plastid gene was inactivated [21]. Therefore, the context between cyanobacteria and chloroplasts appears rather different. In higher plant chloroplasts, the Ndh complex has been exclusively located in stroma lamellae thylakoids [31]. This complex appears to be mainly involved in cyclic electron flow around PSI [21,30] and following inactivation of one of the *ndh* genes, the whole complex disappears. In *Synechocystis*, the Ndh complex can be found both in cytoplasmic membranes and in thylakoid membranes. It is likely involved in respiration and in cyclic electron flow around PSI. Some *ndh* genes are essential, while inactivation of other *ndh* genes leads to a high CO_2 requiring phenotype. Whether such differences in physiological functions and location correspond to differences in subunit composition will require further work to be clearly established.

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