Properties of Mutants of *Synechocystis* sp. Strain PCC 6803 Lacking Inorganic Carbon Sequestration Systems

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A mutant (∆5) of *Synechocystis* sp. strain PCC 6803 constructed by inactivating five inorganic carbon sequestration systems did not take up CO₂ or HCO₃⁻ and was unable to grow in air with or without glucose. The ∆4 mutant in which BicA is the only active inorganic carbon sequestration system showed low activity of HCO₃⁻ uptake and grew under these conditions but more slowly than the wild-type strain. The ∆5 mutant required 1.7% CO₂ to attain half the maximal growth rate. Electron transport activity of the mutants was strongly inhibited under high light intensities, with the ∆5 mutant more susceptible to high light than the ∆4 mutant. The results implicated the significance of carbon sequestration in dissipating excess light energy.

**Keywords:** CO₂ and HCO₃⁻ uptake — CO₂-concentrating mechanism (CCM) — Cyanobacteria — Electron transport — Inorganic carbon sequestration.

Abbreviations: CCM, CO₂-concentrating mechanism; Ci, inorganic carbon; (r)ETR, (relative) electron transport rate; H, high CO₂-grown; L, low CO₂-induced; PAR, photosynthetically active radiation; WT, wild type.

Introduction

Cyanobacteria possess a CO₂-concentrating mechanism (CCM) that involves the uptake of CO₂ and HCO₃⁻ driven by light energy (Giordano et al. 2005, Kaplan et al. 2008, Price et al. 2008). Two CO₂ uptake systems and three HCO₃⁻ transporters have been identified in cyanobacterial strains. These are (i) the low CO₂-inducible high affinity CO₂ uptake system dependent on NdhD3/NdhF3/CupA/CupS (Shibata et al. 2001, Maeda et al. 2002, Zhang et al. 2004, Folea et al. 2001, Shibata et al. 2002); (ii) the constitutively expressed low affinity CO₂ uptake system dependent on NdhD4/NdhF4/CupB (Shibata et al. 2001, Maeda et al. 2002, Xu et al. 2008); the low CO₂-inducible high affinity HCO₃⁻ transporters dependent on (iii) CmpA/CmpB/CmpC/CmpD (Omata et al. 1999, Koropatkin et al. 2007) and (iv) SbtA (Shibata et al. 2002); and (v) the low affinity HCO₃⁻ transporter dependent on BicA (Price et al. 2005). Both CO₂ and HCO₃⁻ are taken up by these systems and are accumulated within the cells as HCO₃⁻, which enters into carboxysomes and is converted to CO₂ by the action of carbonic anhydrase to raise the CO₂ concentration in the vicinity of Rubisco (Kaplan et al. 2008).

The uptake of CO₂ and HCO₃⁻, and leakage of these inorganic carbon (Ci) species occurs during the operation of the CCM (Tchernov et al. 2003, Kaplan et al. 2008). This carbon cycling maintains an ambient CO₂ concentration substantially above or below that expected at chemical equilibrium with the prevailing HCO₃⁻ concentration in the medium (Tchernov et al. 2003). It has been suggested that the cycling of Ci species may play an important role in protecting the cells from high light stress by dissipating excess light energy (Kaplan et al. 2008).

In an attempt to see the significance of Ci sequestration for dissipation of excess energy in cyanobacterial cells, we constructed a mutant (∆4) of *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis* PCC 6803) by inactivating the *bicA* gene (*sll0834*) in the ∆4 mutant (∆*ndhD3/D4/sbtA/cmpA*; Shibata et al. 2002). The ∆5 mutant did not have any Ci sequestration activity. Analyses of the growth and other physiological characteristics of the ∆4 and ∆5 mutants under various conditions revealed a role for Ci sequestration in dissipating excess light energy required for cyanobacterial cells to survive under diverse environmental conditions.

**Results**

**CO₂ and HCO₃⁻ uptake**

The activities of CO₂ and HCO₃⁻ uptake were low in high CO₂-grown (H) cells of the wild type (WT) and were increased 3–4 times in low CO₂-induced (L) cells. We did not detect any activities of CO₂ and HCO₃⁻ uptake in either H-cells or L-cells of the ∆5 strain. This indicates that there are no more than five Ci sequestration systems in *Synechocystis* PCC 6803 functional under the experimental
conditions used here. The BicA-dependent HCO$_3^-$ transporter is the only active Ci sequestration system in the Δ4/C14 strain. It has been reported that this HCO$_3^-$ transporter is induced under low CO$_2$ and is a major contributor to HCO$_3^-$ uptake in a marine cyanobacterium, *Synechococcus* sp strain PCC 7002 (Price et al. 2005). However, the activity of HCO$_3^-$ uptake of the Δ4 mutant was 25% that of the WT in H-cells and was only 6% in L-cells; the activity was not increased by bubbling H-cells of the Δ4 mutant with air overnight (Fig. 1). As expected, the Δ4 mutant did not have CO$_2$ uptake activity.

**Effect of CO$_2$ concentration on growth rate**

The Δ5 mutant of *Synechocystis* PCC 6803 did not grow under air levels of CO$_2$. The Δ4 mutant grew under these conditions, indicating that carbon supply by the BicA HCO$_3^-$ transporter supported the growth of this mutant. However, the growth rate of the Δ4 mutant was less than half that of the WT. The Δ4 and Δ5 mutants required about 1.3 and 1.7% CO$_2$, respectively, to attain half the maximal rate (Fig. 2). The maximal growth rate of these mutants was attained at 2 and 3% CO$_2$, respectively, where the growth is supported by passive diffusion of Ci, predominantly in the Δ4 mutant and totally in the Δ5 mutant. The WT cells grew more slowly under air than under high CO$_2$ concentrations. The growth rate started to increase at around 0.6% CO$_2$ to reach the maximal level at 1% CO$_2$, where the growth rate of the Δ5 mutant was nearly zero.

**Growth under photonPhotomixotrophic conditions**

*Synechocystis* PCC 6803 used to construct the mutants is the strain that is able to utilize glucose and to grow under photomixotrophic conditions as well as under photoautotrophic conditions (Fig. 3A, curves b and a, respectively). It was assumed that the Δ5 mutant was also able to grow in air using glucose as a carbon source. However, the mutant which was unable to grow under bubbling with air died after a certain period of slow growth in the presence of glucose (Fig. 3C, curves e and f, respectively). The mutant grew under high CO$_2$ (curve g), and glucose accelerated the growth under these conditions (curve h). The Δ4 strain grew slowly under air, and glucose accelerated the growth (curves c and d), but to a level lower than that of the WT (curve b).

**Effect of light intensity on relative electron transport rate (rETR)**

Fig. 4 shows the ETR (relative) values obtained for the WT and mutant cells under various light intensities.
At light intensities below 100 \( \text{mE/m}^2\text{s/cm}^2 \) photosynthetically active radiation (PAR), there was no significant difference between the WT and mutant strains in their ETR. The ETR values increased in the WT by increasing the light intensity to attain the maximal value at around 750 \( \text{mE/m}^2\text{s/cm}^2 \) and then decreased at higher intensities. In contrast, ETR values in the \( \Delta 4 \) and \( \Delta 5 \) mutants decreased at light intensities above 100 \( \text{mE/m}^2\text{s/cm}^2 \) to reach zero value at 460 and 720 \( \text{mE/m}^2\text{s/cm}^2 \), respectively. The \( \Delta 5 \) mutant was more susceptible to high light than the \( \Delta 4 \) mutant at the intensities above 50 \( \text{mE/m}^2\text{s/cm}^2 \).

**\( \Delta pH \) build-up**

Fig. 5 shows the time courses of build-up of the proton gradient across the thylakoid membranes of the WT and mutants, as measured by acridine yellow fluorescence. Kinetic parameters of the proton gradient build-up, calculated from the curves as shown in Fig. 5, are summarized in Table 1. These results indicated the build-up of the \( \Delta pH \) gradient in the mutants as well as in the WT, although this was significantly slower in the mutants, decreasing from \( \Delta 4 \) to \( \Delta 5 \) mutants. However, the equilibrium \( \Delta pH \) gradient (shown by the \( \Delta F/F \) ratio at the steady-state level) was higher in the \( \Delta 4 \) mutant and lower in the \( \Delta 5 \) mutant, respectively, as compared with that in the WT (Table 1).

**Discussion**

The present study clearly showed that *Synechocystis PCC 6803* does not possess more than five Ci sequestration systems functional under the experimental conditions used here, since inactivation of all of these systems in the \( \Delta 5 \) mutant completely abolished the activity of CO\(_2\) and

### Table 1 Kinetic parameters of the \( \Delta pH \) build-up in WT and mutant *Synechocystis PCC 6803*

<table>
<thead>
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<th>( t_{1/2} ) (s)</th>
<th>( \Delta F/F )</th>
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<tbody>
<tr>
<td>WT</td>
<td>20.5 ± 1.6</td>
<td>1.45 ± 0.09</td>
</tr>
<tr>
<td>( \Delta 4 )</td>
<td>57 ± 8</td>
<td>2.13 ± 0.09</td>
</tr>
<tr>
<td>( \Delta 5 )</td>
<td>73 ± 13</td>
<td>1.09 ± 0.21</td>
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*Expressed as mean±SD; individual data (n=4 independent cultures) were obtained from acridine yellow experiments as shown in Fig. 5.*
HCO\textsubscript{3}\textsuperscript{-} uptake (Fig. 1). The mutant was unable to grow under air levels of CO\textsubscript{2} whereas the Δ4 mutant, where the BiC\textsubscript{A} HCO\textsubscript{3}\textsuperscript{-} transporter is the only active Ci sequestration system, grew under air but much more slowly than the WT (Fig. 2). Thus, under air levels of CO\textsubscript{2}, the growth is limited by the supply of carbon in the Δ4 mutant. The growth rate of the WT in air was lower than that under high CO\textsubscript{2} concentrations; the maximal growth rate was attained at 1% CO\textsubscript{2} where the growth of the Δ5 mutant was nearly zero (Fig. 2). Under air levels of CO\textsubscript{2}, all of the five Ci sequestration systems are expressed in the WT and, therefore, its growth may not be limited by the availability of carbon. This is supported by the observation of leakage of CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-} during uptake of these species under air levels of CO\textsubscript{2} by the Ci sequestration systems (TeChernov et al., 2001; Tchernov et al., 2003). On the other hand, a considerable level of glycolate is produced under these conditions but not under high CO\textsubscript{2}, suggesting that Rubisco is not saturated with CO\textsubscript{2} under air levels of CO\textsubscript{2} (Colman, 1989, Eisenhut et al. 2006). It is possible that, although the WT strain takes up Ci in excess under these conditions, the rates of transfer of HCO\textsubscript{3}\textsuperscript{-} accumulated within the cytoplasm and conversion to CO\textsubscript{2} in the carboxysomes are not high enough to saturate Rubisco with CO\textsubscript{2}. The lower growth rate of the WT at CO\textsubscript{2} concentrations below 1% might be attributed to the following reasons: (i) Rubisco is not saturated with CO\textsubscript{2} under low CO\textsubscript{2} as discussed above; (ii) changes in cell physiology related to the down-regulation of expression of many genes under low CO\textsubscript{2} such as those encoding ribosomal proteins (Wang et al., 2004); and/or (iii) a significant portion of the available energy is consumed for Ci uptake under low CO\textsubscript{2} at a relatively low light intensity of 50 μE m\textsuperscript{-2} s\textsuperscript{-1} and limitation in energy retards the growth. The Δ5 mutant, in which CO\textsubscript{2} is supplied from the external medium by diffusion, possibly through a water channel (Tchernov et al., 2001), required about 1.7% CO\textsubscript{2} to attain half the maximal growth rate (Fig. 2). The concentration of CO\textsubscript{2} in water at 30°C in equilibrium with 1.7% CO\textsubscript{2} in air is calculated to be 455 μM, which is much higher than the K\textsubscript{m}(CO\textsubscript{2}) value of Rubisco of *Synechocystis* PCC 6803 (=162–202 μM; Marcus et al., 2003). In this mutant, CO\textsubscript{2} enters the cells only by diffusion. The requirement for a CO\textsubscript{2} concentration for growth much higher than that expected from the K\textsubscript{m}(CO\textsubscript{2}) value of Rubisco indicates the presence of high resistance to CO\textsubscript{2} diffusion at the site of entry to the cells and/or to the carboxysomes.

One of the most significant findings of this study is that both Δ4 and Δ5 mutants are susceptible to high light intensities (Fig. 4). Previous data of Helman and co-workers (2005) show that electron flow to O\textsubscript{2} can reach up to 40% of the photosynthetic electron flow originating from PSII in cyanobacteria. Also, genes encoding A-type flavoproteins involved in photoreduction of O\textsubscript{2} have been identified (Helman et al. 2003). In the Δ5 and Δ4 mutants, most of the electrons leaving PSII may be used to reduce O\textsubscript{2} due to the absence or low activity of carbon uptake and fixation (Fig. 1). This might cause damage to the cells at high light intensities when electron supply by PSII is increased.

Excess electrons from PSII inhibit their electron transport activity by fully reducing the electron transport chain and causing photodamage, especially in the mutants where there is no or a highly limited amount of carbon supply (Fig. 1). The Δ5 mutant was unable to grow under air even in the presence of glucose, and the Δ4 mutant grew slowly under air and glucose accelerated the growth, but to a level lower than that observed with the WT (Fig. 3). The result supports the above view that excess electrons (from PSII and glucose) inhibit the electron transport activity. The growth curves were obtained at 50 μE m\textsuperscript{-2} s\textsuperscript{-1} where the inhibition of ETR was not observed (Fig. 4). However, the growth is highly dependent on the availability of Ci species, which are much lower in the mutants than in the WT (Fig. 1).

The trans-thylakoid proton gradient build-up is highly dependent on the ETR and CCM (Teuber et al., 2001, Berry et al. 2005). The observation that the proton gradient is formed in the mutants indicates the presence of electron transport activity even at the high light intensity (1,700 μE m\textsuperscript{-2} s\textsuperscript{-1}) where the apparent rETR value was nearly zero (Figs. 4, 5). It is possible that the formation of ΔPH in the mutants depends mostly on cyclic electron transport and/or there is a certain linear electron transport activity even when plastoquinone is highly reduced (giving a very low apparent rETR value). While linear and cyclic electron transport are directly coupled to proton transduction (build-up of ΔPH), ATP synthesis essential for CO\textsubscript{2} fixation is driven by a pH gradient. The steady-state ΔPH level is determined by the balance of these opposing processes. The larger ΔF/ΔF\textsuperscript{o} ratio of the Δ4 mutant in spite of the slower ΔPH formation, as compared with that in the WT (Fig. 5 and Table 1), suggests that the utilization of the proton gradient is more limited in the Δ4 mutant than the ΔPH formation by electron transport processes. The low ΔF/ΔF\textsuperscript{o} ratio in the Δ5 mutant indicates that in this strain, the build-up of ΔPH is very low, most probably because of the low electron transport activity.

The way by which the Ci sequestration systems are energized is not known, except for the ABC-type HCO\textsubscript{3}\textsuperscript{-} transporter (Omata et al. 1999). It has been reported that CO\textsubscript{2} uptake systems are energized by PSI-dependent cyclic electron flow whereas HCO\textsubscript{3}\textsuperscript{-} transport is dependent on linear electron transport (Li and Canvin 1998). The uptake and leakage of CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-} occur simultaneously during the operation of the CCM (Tchernov et al., 2003, Kaplan et al. 2008), producing a cycling of Ci species. It has been suggested that this carbon cycling may play an
important role in protecting the cells from high light stress by dissipating excess light energy (Tchernov et al. 2003, Kaplan et al. 2008). Our present work provided the experimental evidence to support this hypothesis.

Materials and Methods

Growth conditions
WT and mutant cells of Synechocystis PCC 6803 were grown at 30°C in BG11 medium (Stanier et al. 1971) buffered with 20 mM TES-KOH (pH 8.0) and bubbled with ambient (with about 380 p.p.m. CO₂) air under continuous illumination of 50 µE m⁻² s⁻¹ PAR provided by white fluorescent lamps.

Construction of mutants
Construction of the ΔndhD3/ndhD4/cmpA/shtA (designated Δ4) mutant has been described previously (Shibata et al. 2002) and deposited in the web site CyanoMutants (http://www.kazusa.or.jp/cyano/mutants/). The cassettes used to inactivate ndhD3, ndhD4, cmpA and shaltA are those which give resistance to spectinomycin, kanamycin, hygromycin and chloramphenicol, respectively. A gentamycin-resistant cassette was used to inactivate bicA (sll0834) in the Δ4 mutant to create the Δ5 mutant. The DNA region containing bicA was amplified by PCR using the following primers: 68BicEcoF, GGGGAATTCGGTGGTTGGTTAGA; and 68BicR, TCATTCAAGCCATCAGC. The PCR product was digested with EcoRI and HindIII, and cloned into pUC18. The gentamycin cassette was then inserted into the PstI site inside bicA and the plasmid thus constructed was used to transform the Δ4 mutant to generate the Δ5 mutant in which all of the five Ci uptake systems were inactivated. PCR analysis of the mutant confirmed complete segregation of the inactivated bicA.

Determination of growth rates
H-cells from WT and mutant strains were collected by centrifugation at 3,000 g for 5 min and resuspended in fresh BG11 medium to an OD_{730} of 0.1. Glucose (5 mM) was added for photomixotrophic growth. Tubes containing 50 ml of the cell suspension were placed at 30°C and aerated with CO₂-enriched (3% v/v) or ambient air under continuous illumination of 50 µE m⁻² s⁻¹ PAR. The OD_{730} was measured using a spectrophotometer, model UV755B (MC Scientific Instruments, Shanghai, PR China).

Measurement of CO₂ uptake
WT and mutant H- and L-cells (the latter bubbled with air for 18 h) were harvested by centrifugation and suspended in 25 ml of 20 mM HEPES-KOH pH 7.0 supplemented with 15 mM NaCl at a chlorophyll level of 5.0 µg ml⁻¹, and placed in a reaction vessel (Ogawa et al. 1985). CO₂ exchange of the cell suspension was measured at 30°C using an open gas analysis system, which measures the rate of CO₂ exchange as a function of time. Ambient air containing 377–380 p.p.m. CO₂ was passed into the reaction vessel at a flow rate of 1.0 liter min⁻¹, the exchanged gas was dried, and then the CO₂ concentration was analyzed using an infrared gas analyzer, model WMA-4 (PP Systems, Amesbury, MA, USA).

Measurement of HCO₃⁻ uptake
The rate of HCO₃⁻ uptake was measured using H¹⁴CO₃⁻ in BG11 medium (pH 8.0) which had been bubbled with ambient air (~380 p.p.m. CO₂) for several hours. Cells were suspended in the aerated BG11 and 1 ml of cell suspension was mixed with an equal volume of the aerated BG11 containing NaH¹⁴CO₃ and then 0.9 ml of the mixture was taken up by an Eppendorf pipet. Ci uptake was initiated by illuminating the cells in the pipet by white light (800 µE m⁻² s⁻¹) and terminated after 30 s by rapid filtration of the cells onto a glass filter (GF/B, Whatman) by suction, followed by immediate washing of the filter with 5 ml of BG11 medium. The filter was washed once more and then subjected to the measurement of radioactivity.

Measurement of rETR
Changes in chlorophyll fluorescence yield in the WT and mutant cells were measured using a Dual-PAM-100 measuring system (Walz, Effeltrich, Germany). The actinic light intensity was increased stepwise from 0 to 1,700 µE m⁻² s⁻¹; steady-state (F) and maximal (F_m) fluorescence levels were determined after 30 s adaptation periods at each light intensity. F_m was obtained by applying 300 ms saturating pulses with an intensity of 10,000 µE m⁻² s⁻¹.

The rETR values were calculated using the following equations as reported by Genty et al. (1989).

\[
ETR = \Phi_{PSII} \text{PAR} a^* n_{PSII}
\]

where \(\Phi_{PSII} = (F_m - F) / F_m\) is the effective PSII quantum yield, \(a^*\) is the PSII (optical) cross-section and \(n_{PSII}\) is the number of PSII centers.

As \(a^*\) and \(n_{PSII}\) can be considered equal in the WT, Δ4 and Δ5 mutants, the rETR can be calculated as

\[
rETR = \text{PAR} (F_m - F) F_m /
\]

ΔpH measurements
Relative pH gradient changes across the thylakoid membranes were determined by using the pH indicator acridine yellow as described by Teuber et al. (2001). Excitation and detection of the fluorescence was done by the Dual-PAM-100 modules DUAL-EAY and DUAL-DAY, respectively, in a 90° arrangement; saturated red light (PAR = 1,700 µE m⁻² s⁻¹) was provided by the LED (light-emitting diode) array of DUAL-DAY.

Other methods
Unless otherwise stated, standard techniques were used for DNA manipulation. Pigments in the cells were extracted in methanol, and the concentration of chlorophyll in the extract was determined (Ogawa and Shibata 1965).

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Mutants lacking inorganic carbon uptake systems

1677

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