Inactivation of \textit{spkD}, encoding a Ser/Thr kinase, affects the pool of the TCA cycle metabolites in \textit{Synechocystis} sp. strain PCC 6803

Sophie Laurent,\textsuperscript{1,2} Jichan Jang,\textsuperscript{2} Annick Janicki,\textsuperscript{2} Cheng-Cai Zhang\textsuperscript{1,2} and Sylvie Bédu\textsuperscript{2}

\textsuperscript{1}Aix-Marseille Université, Institut de Biologie Structurale et Microbiologie, 13402 Marseille cedex 20, France
\textsuperscript{2}CNRS, Laboratoire de Chimie Bactérienne (UPR9043), Institut de Biologie Structurale et Microbiologie, 13402 Marseille cedex 20, France

The inactivation of \textit{sll0776} (\textit{spkD}), a gene encoding a protein Ser/Thr kinase in \textit{Synechocystis} PCC 6803, led to a pleiotropic phenotype of the \textit{SpkD} null mutant. This mutant is impaired in its growth ability under low concentration of inorganic carbon (\textit{C}_i), though its \textit{C}_i-uptake system is not affected. Addition of glucose, phosphoglyceraldehyde or pyruvate does not allow the mutant to grow under low-\textit{C}_i conditions. In contrast, this growth defect can be restored when the low-\textit{C}_i culture medium is supplemented with metabolites of the TCA cycle. Growth of the mutant is also inhibited when ammonium is provided as nitrogen source, whatever the carbon regime of the cells, due to the high demand for 2-oxoglutarate, which is the carbon skeleton for ammonium assimilation. When mutant cells are cultured under standard growth conditions, the intracellular concentration of 2-oxoglutarate is 20\% lower than is observed in the wild-type strain. However, this decrease of 2-oxoglutarate level only slightly affects the phosphorylation state of PII, a protein that regulates nitrogen and carbon metabolism according to the intracellular levels of 2-oxoglutarate. Properties of the \textit{SpkD} mutant suggest that the Ser/Thr kinase \textit{SpkD} could be involved in adjusting the pool of the TCA cycle metabolites according to \textit{C}_i supply in the culture medium.

\textbf{INTRODUCTION}

The cyanobacterium \textit{Synechocystis} sp. strain PCC 6803 is able to adapt to either low or high inorganic carbon (\textit{C}_i) concentrations for autotrophic growth (Ogawa & Kaplan, 2003). It also has the ability, unusual for a photosynthetic organism, to grow under photoheterotrophic and heterotrophic conditions, using glucose as carbon source (Rippka \textit{et al.}, 1979; Zhang \textit{et al.}, 1989). Another unusual characteristic of cyanobacteria is also illustrated by the coexistence of both metabolic pathways characteristic of the so-called \textit{C}_3 and \textit{C}_4 organisms. The \textit{C}_4 acid pathway is a secondary route used by cells to supplement the internal pool of the tricarboxylic acid (TCA) cycle metabolites, via the phosphoenolpyruvate carboxylase (PEPC) activity. Thus, carbon dioxide fixation may occur via the reducing pentose-phosphate pathway, leading to the synthesis of the \textit{C}_3 3-phosphoglyceraldehyde, or via the \textit{C}_4 acid pathway, with carboxylation of phosphoenolpyruvate (PEP) into oxaloacetic acid (Coleman & Colman, 1981).

Regulatory pathways modulating carbon assimilation must be involved in the control of complex metabolic networks in order to balance carbon and carbon/nitrogen metabolic activities. The analysis of transcript changes during long-term \textit{C}_i-limitation in \textit{Synechocystis} PCC 6803 indicated a stable upregulation of genes encoding the inducible \textit{C}_i-uptake systems and enzymes involved in outer cell wall polysaccharide synthesis (Eisenhut \textit{et al.}, 2007). However, only a few regulatory elements that control carbon metabolism have been identified. Two well-studied proteins, PII and NtcA, are known to be involved in the regulation of the balance between carbon and nitrogen metabolisms, although their major functions appear to be to control nitrogen metabolism (Herrero \textit{et al.}, 2001; Forchhammer, 2004). The activity of both NtcA and PII is modulated by 2-oxoglutarate, whose intracellular concentration reflects the carbon/nitrogen status in cyanobacteria (Muro-Pastor \textit{et al.}, 2001; Laurent \textit{et al.}, 2005). In \textit{Synechococcus} PCC 7942, a complex of PII–2-oxoglutarate leads to the phosphorylation of PII by an unknown protein kinase (Irmler \textit{et al.}, 1997). In \textit{Synechocystis} PCC 6803, PII dephosphorylation under conditions of low 2-oxoglutarate levels is catalysed by PphA, a PP2C-type protein phosphatase (Ruppert \textit{et al.}, 2002). One His-kinase, Hik8, has been characterized as essential for the regulation of heterotrophic growth (Singh & Sherman, 2005). A PP2C-type

\textbf{Abbreviations:} \textit{C}_i, inorganic carbon; LC, low-\textit{C}_i; PEPC, PEP carboxylase; SC, standard \textit{C}_i; TCA, tricarboxylic acid.
protein phosphatase, IcfG, was identified as being involved in co-ordinated regulation of C_i and glucose metabolism (Beuf et al., 1994; Shi et al., 1999).

*Synechocystis* PCC 6803 contains seven ORFs encoding Ser/Thr kinases (Leonard et al., 1998, Zhang et al., 1998). Two Ser/Thr kinases, SpkA and SpkB, are involved in the regulation of cell motility (Kamei et al., 2001, 2002). The functions of the other Ser/Thr kinases remain unknown although their Ser/Thr kinase activity has been tested *in vitro* (Kamei et al., 2003). We have constructed insertional mutants inactivating all seven Ser/Thr kinase genes and analysed their capability to adapt to different carbon regimes. In this study, we show that a *sll0776* null mutant presents a pleiotropic phenotype, and particularly, cannot grow under a low-C_i regime or standard C_i regime when NH_4^+ is the nitrogen source. Based on our results, we propose that the Ser/Thr kinase encoded by *sll0776* (named as *spkD* by Kamei et al., 2002) is involved in the regulation of the pool of the TCA cycle metabolites.

**METHODS**

**Strains and growth conditions.** *Synechocystis* PCC 6803 was obtained from the Pasteur Culture Collection, and cultured in modified Allen’s medium (Bedu et al., 1995). Two C_i regimes were tested, a low-C_i regime (LC), medium buffered with 20 mM HEPES pH 8.2 with C_i as the sole C_i source, or a standard C_i regime (SC), medium containing 12 mM bicarbonate, pH 8.2. For photoheterotrophic conditions, glucose was added at a final concentration of 20 mM and a final concentration of 20 mM. When indicated, 5 mM was added to the growth medium instead of nitrate as the nitrogen source.

**Construction of Δ0776 mutant.** A 1.3 kb DNA fragment including the *sll0776* coding region was amplified by PCR from the genomic DNA of *Synechocystis* PCC 6803 using the following primers: 5'-ATTGAATGTCAAGTACTCGACCGTT-3' and 5'-GGATTTCCTCGAATGTTCGGCTACACC-3'. The amplified DNA fragment was cloned in a pUCBM20 vector (Boehringer Mannheim). The mutant was constructed by insertion of a 1.2 kb kanamycin resistance cassette cloned in a pUCBM20 vector (Boehringer Mannheim). The mutant was confirmed by PCR.

**RT-PCR analysis.** Total RNA was isolated from cells adapted to SC or LC culture conditions and treated with Amplification-grade DNase I (Invitrogen). Reverse transcription was performed with the Superscript II RT/Platinium Taq System (Invitrogen), using primers as shown in Fig. 1. *rpnB* was used as a control for the amount of RNA used in each essay. As a control, RT-PCR was carried out by omitting the reverse transcriptase; this confirmed that the RNA extracts were not contaminated by chromosomal DNA (data not shown).

**Measurements of C_i uptake and enzyme activities.** C_i uptake was measured as described by Bedu et al. (1995), using a concentration range of 10 to 500 μM of NaH^{14}CO_3. Cells were grown to OD_{580} = 1 and then concentrated threefold. After 20 s incubation with NaH^{14}CO_3, the cell suspension was filtered on glass fibre filters and the retained radioactivity was measured.

**RESULTS AND DISCUSSION**

**Characteristics of SpkD (Sll0766) and genomic organization of the sll0776 gene cluster**

SpkD, encoded by the ORF *sll0766* in *Synechocystis* PCC 6803, presents three distinct domains (Fig. 1a): a conserved Ser/Thr kinase catalytic domain (S/T K) and a SH3 domain, separated by a putative transmembrane segment (TM). (b) Organization of the *sll0776* gene cluster; arrowheads indicate the pairs of PCR primers used to amplify the intergenic regions of the cluster. (c) Gene expression in cells grown under SC (12 mM HCO_3^-) or LC (CO_2 as the sole carbon source) medium, analysed by semi-quantitative RT-PCR, with the expression of *rpnB* as a control.

For determination of enzymic activities, cells were broken with glass beads and protein extracts were cleared by centrifugation for 10 min at 20,000 g. Pyruvate dehydrogenase activity was determined spectrophotometrically at 340 nm, measuring the reduction of NAD^+, according to Paurding et al. (2001); the reactions were started by addition of pyruvate at 10 mM. The PEPC activity was measured spectrophotometrically at 340 nm, coupling the reaction to NADH oxidation mediated by malate dehydrogenase, as described by Le Van Quy et al. (1991); the reactions were started by addition of PEP at 2.5 mM.

**Determination of the intracellular 2-oxoglutarate levels.** Cell suspension (30 ml) at OD_{580} = 1 was collected by rapid filtration under vacuum, using an 85 mm diameter membrane filter with a 0.45 μm pore size (Schleicher & Schuell) under illumination (40 μE), and lysed with cold 0.3 M HClO_4. The lysate was centrifuged and the supernatant neutralized with 2 M K_2CO_3. The 2-oxoglutarate concentration in the supernatant was determined using a glutamate dehydrogenase assay (Sigma) according to the manufacturer’s instructions.

**Other techniques.** PII modifications were analysed using native gel electrophoresis coupled to immunoblot techniques according to Forchhammer & Tandeau de Marsac (1994). Phycobiliproteins were determined as described by Collier & Grossman (1992).

---

![Fig. 1. The SpkD protein and the expression of *sll0775*, *sll0776* and *sll0777*.](image-url)
transmembrane domain, and an SH3 domain at the C terminus. SH3 domains are found in many signalling proteins in eukaryotes, a number of them being protein kinases (Ponting et al., 1999), especially Src protein-tyrosine kinases (Roskoski, 2004). SH3 domains often interact with proline-rich protein domains (Yu et al., 1994). SpkD is currently the only prokaryotic protein showing a combination of a Ser/Thr-kinase domain and an SH3 domain.

The sll0776 gene encoding SpkD is part of a gene cluster including sll0775, sll0777 and sll0778 (Fig. 1b). sll0775 encodes a protein with no sequence similarity to others found in the databases. sll0777 encodes a membrane protein with an N-terminal hydrophobic segment, bearing two domains: a carboxypeptidase domain and a proline-rich domain. sll0777 may also be a membrane protein with five putative transmembrane segments; its amino acid sequence presents high similarity to an ABC transporter including sll0775 and sll0777. sll0775 and sll0777 were not detected under the growth conditions tested.

**sll0776 is essential for cell growth under low carbon regime**

A sll0776 null mutant (Δ0776) was constructed by insertion of a kanamycin-resistance cassette into a Bgl site, located in the region corresponding to the conserved catalytic domain of protein kinases (Fig. 1a). PCR analysis indicated that the mutant was completely segregated (data not shown). The growth rate of Δ0776 cultured under SC conditions (Fig. 1c). sll0776 was observed whatever the carbon regimes, while expression of sll0775 was not detected under the growth conditions tested.

We determined the threshold HCO$_3^-$ concentration that triggered growth of the mutant. The addition of 10 or 50 μM HCO$_3^-$ in LC culture medium did not allow the growth of the mutant; however, when the concentration of HCO$_3^-$ was increased to 100 μM the mutant started to grow. This concentration is close to the apparent $K_m$ (60 ± 12 μM), evaluated for the high-affinity transport system of HCO$_3^-$ activated under LC growth conditions (Bédou et al., 1995). Therefore, the HCO$_3^-$ uptake activity was measured and the results obtained demonstrated that both wild-type and mutant cells grown under either SC or LC regime for 6 h displayed similar HCO$_3^-$ uptake activities, even with a slight stimulation in the mutant (Fig. 3). These results indicated that the phenotype observed in the mutant was not caused by a deficiency in C$_4$ uptake.

**The growth defect of the sll0776 mutant can be rescued by addition of metabolites of the TCA cycle**

The addition of glucose (20 mM), with or without DCMU (10 μM), to the growth medium did not compensate the growth defect of the mutant under LC growth conditions. The same results were obtained after addition of 20 mM phosphoglyceraldehyde or pyruvate (Table 1). However, when LC medium was supplemented with TCA cycle metabolites (20 mM), such as acetyl-CoA, succinate, citrate or 2-oxoglutarate, the growth capacity of the Δ0776
mutant was restored (Table 1). These observations suggest that the growth impairment of the mutant is not due to a deficiency in the C$_3$-assimilation process itself, including the Calvin cycle pathway, but rather due to a deficiency at the level of the TCA cycle anabolic pathway (summarized in Fig. 4). This idea is consistent with the results obtained when we measured the activity of pyruvate dehydrogenase. This enzyme catalyses the formation of acetyl-CoA, providing the entry point into the TCA cycle. No significant differences were detected between the wild-type and the mutant grown under either LC or SC growth conditions (Fig. 5a). These results indicate that the impairment of the pool of the TCA cycle metabolites did not originate at this step of the anabolic pathway.

Other properties of the mutant support the hypothesis of a deficiency in the pool of TCA cycle metabolites. First, we determined the intracellular concentration of 2-oxoglutarate of cells grown under SC conditions and observed a reduction of 20% in the Δ0776 mutant $[3.5 \pm 0.3 \text{ nmol (mg protein)}^{-1}]$ in comparison with that found in the wild-type strain $[4.6 \pm 0.2 \text{ nmol (mg protein)}^{-1}]$. Secondly, we

<table>
<thead>
<tr>
<th>Addition to LC growth conditions</th>
<th>Wild-type</th>
<th>Δ0776 mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Phosphoglyceraldehyde</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Succinate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 3. HCO$_3^-$ uptake activities in the wild-type (■) and the Δ0776 mutant (●), grown for 6 h under LC conditions (a) or adapted to the SC conditions (b). Cells were incubated with 10 to 500 μM H$^{13}$CO$_3$.

Fig. 4. Summary of the metabolites restoring (dark grey shading) or not restoring (light grey shading) the Δ0776 mutant growth under LC culture conditions. PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PGA, phosphoglycerdehyde.

Fig. 5. Pyruvate dehydrogenase (a) and phosphoenolpyruvate carboxylase (b) activities, measured in the wild-type (WT) and Δ0776 mutant grown under SC (dark grey bars) and LC (light grey bars) conditions.
observed that the Δ0776 mutant did not grow when ammonium (5 mM) was present as a nitrogen source, whatever the carbon regime for the cells (Fig. 2b). Several studies have demonstrated that the intracellular levels of the TCA cycle metabolites, and in particular those of 2-oxoglutarate, dramatically drop when ammonium instead of nitrate is the nitrogen source in Synechocystis PCC 6803 (Mérida et al., 1991; Muro-Pastor et al., 2001). Indeed, ammonium is readily incorporated into the carbon skeleton for amino acid synthesis while nitrate needs to be reduced first into ammonium. The pool of the TCA cycle metabolites in the Δ0776 mutant may be too low to meet the high demand created by the presence of ammonium, making the cells unable to grow with this nitrogen source. Addition of 20 mM 2-oxoglutarate in SC medium containing ammonium allowed the mutant cells to grow and confirmed this hypothesis.

**Pleiotropic aspects of the Δ0776 mutant phenotype**

As already mentioned, carbon limitation triggers several cell acclimation responses. Among them are the activation of the C₄ acid pathway to compensate the lower carbon supply (Tabita, 1994), the degradation of phycobiliproteins as a reserve material (Schwarz & Grossman, 1998) and the dephosphorylation of regulatory protein PII (Hisbergues et al., 1999). We thus examined each of these aspects in both the wild-type and the mutant.

The C₄ acid pathway accounts for 20 to 60% of carbon supply in cyanobacteria (Tabita, 1994; Yang et al., 2002; Colman et al., 1976) and the major enzyme of this pathway, the PEP carboxylase (PEPC), is inhibited when the concentration of the TCA cycle intermediates, such as malate, is high (Owttrim & Colman, 1988). The activity of plant PEPCs is controlled by reversible phosphorylation, the phosphorylated form of the enzyme being the active one. Although post-translational modifications of cyanobacterial PEPCs have not been shown (Chollet, 1996), we measured the PEPC activity on crude cell extracts from the wild-type and the Δ0776 mutant. The results revealed a threefold increase in the enzyme activity in the mutant compared to the wild-type strain, whatever the C₄ regime of the cells (Fig. 5b). The stimulation of PEPC activity correlates well with a lower level of the TCA cycle metabolites in the mutants. Yang et al. (2002) demonstrated that the C₄ pathway in cyanobacteria involves both PEPC and malic enzyme. In their metabolic fluxes analysis, they showed a substantial output flow from the TCA cycle from malate to pyruvate, driven by the malic enzyme, resulting in CO₂ evolution and NADPH production. The low level of TCA cycle metabolites in the mutant may impair the malic enzyme activity and consequently reduce the synthesis of reducing equivalent (NADPH) and CO₂ evolution.

Phycobiliproteins are used by cyanobacteria as a nutrient reserve and can be degraded under different nutrient starvation conditions, including carbon limitation (Schwarz & Grossman, 1998). While the wild-type cells of Synechocystis PCC 6803 still degraded phycobiliproteins under LC growth conditions, no such degradation was observed in the mutant grown under the same conditions (Fig. 6); however, the degradation of phycobiliproteins still occurred in this mutant under nitrogen starvation, as in the wild-type (data not shown).

The regulatory PII protein is a sensor of 2-oxoglutarate and can be modified in unicellular cyanobacteria according to the intracellular concentration of 2-oxoglutarate (Forchhammer, 2004). When its concentration is high, 2-oxoglutarate forms a complex with the PII protein, leading to the phosphorylation of PII by an unknown protein kinase. We examined whether the reduced intracellular concentration of 2-oxoglutarate observed in the Δ0776 mutant under SC conditions could affect the level of PII phosphorylation. When cells were grown under standard conditions, the level of PII phosphorylation in the mutant was barely reduced compared to the wild-type strain (Fig. 7, lane 1). Muro-Pastor et al. (2001) observed that when cells were shifted from nitrate to ammonium regime, which normally leads to PII dephosphorylation (Forchhammer & Tandeau de Marsac, 1994), the intracellular concentration of 2-oxoglutarate dropped by 80%. Our results indicated that the 20% decrease in the intracellular 2-oxoglutarate concentration in the mutant grown under standard conditions may not be sufficient to prevent efficiently the formation of 2-oxoglutarate–PII complex and consequently does not inhibit the activity of the unknown kinase responsible for the PII protein phosphorylation. Moreover, these results clearly show that SpkD is not this unknown kinase. The dephosphorylated state of PII after 1 h incubation under ammonium regime is presented in Fig. 7, lane 4. When cells were shifted from SC to LC growth conditions (Fig. 7, lanes 2 and 3) the phosphorylation state of PII was only slightly reduced in the wild-type strain; in the Δ0776 mutant, in addition to a reduction in the amount of PII we observed a significant demodification of the protein. This is in good agreement with the proposal of a drastic drop of intracellular concentration of TCA cycle metabolites, specifically 2-oxoglutarate, when mutant cells are shifted from SC to LC growth conditions.

![Fig. 6](http://mic.sgmjournals.org)

**Fig. 6.** Phycocyanin to chlorophyll a (PC: Chl) ratio determined in cells shifted from SC to LC growth conditions after 18 h. ■, Wild-type; ●, Δ0776 mutant.
The analysis of the Δ0776 mutant phenotype highlights the complexity of the regulatory network involved in keeping the balance among various activities of carbon metabolism as well as between carbon and nitrogen metabolism. On the one hand, we observed that the Δ0776 mutant remains able to activate the high-affinity HCO$_3^-$-uptake system and the C$_4$ acid pathway, indicating that the SpkD kinase is not involved in the recognition of or the response to the low carbon signal(s) regulating these metabolic functions. On the other hand, our results indicated that SpkD could be involved in the regulatory pathways leading to the adjustment of the pools of the TCA cycle metabolites and the degradation of phycobiliproteins as a carbon reserve. An open question raised by these puzzling observations is whether the adjustment of the pool of the TCA cycle intermediates and the phycobiliprotein degradation are under the control of a common signalling system of low-C$_4$ perception in which SpkD may have a central function.

ACKNOWLEDGEMENTS

This work was supported by the Centre National de la Recherche Scientifique. S.L. was supported by a fellowship from the Ministry of Education, France, and J.J. by a fellowship from Wonbongunique Co. Ltd, Republic of Korea.

REFERENCES


Edited by: K. Forchhammer