

## Identification of Two Genes, *sl10804* and *slr1306*, as Putative Components of the CO<sub>2</sub>-Concentrating Mechanism in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803<sup>∇</sup>

Shulu Zhang, Kevin W. Spann, Laurie K. Frankel, James V. Moroney, and Terry M. Bricker\*

*Department of Biological Sciences, Biochemistry and Molecular Biology Section, Louisiana State University, Baton Rouge, Louisiana 70803*

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**Insertional transposon mutations in the *sl10804* and *slr1306* genes were found to lead to a loss of optimal photoautotrophy in the cyanobacterium *Synechocystis* sp. strain PCC 6803 grown under ambient CO<sub>2</sub> concentrations (350 ppm). Mutants containing these insertions (4BA2 and 3ZA12, respectively) could grow photoheterotrophically on glucose or photoautotrophically at elevated CO<sub>2</sub> concentrations (50,000 ppm). Both of these mutants exhibited an impaired affinity for inorganic carbon. Consequently, the *Sl10804* and *Slr1306* proteins appear to be putative components of the carbon-concentrating mechanism in *Synechocystis* sp. strain PCC 6803.**

The growth rate of cyanobacteria depends on the amount of available inorganic carbon (C<sub>i</sub>), i.e., CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>. An effective photosynthetic carbon-concentrating mechanism (CCM) has evolved to elevate the CO<sub>2</sub> concentration in the vicinity of the CO<sub>2</sub>-fixing enzyme Rubisco. This enables efficient CO<sub>2</sub> fixation despite Rubisco's inherent low affinity for CO<sub>2</sub> (12). Genetic and physiological studies of cyanobacterial mutants that require high CO<sub>2</sub> concentrations for growth have identified a number of genes directly related to the CCM (11, 12, 15).

*Synechocystis* sp. strain PCC 6803 is a unicellular, naturally transformable cyanobacterium that can grow photoheterotrophically in the presence of glucose (4, 14, 17). This cyanobacterium has proved to be an ideal model organism for study of the mechanism and regulation of oxygenic photosynthesis (5). Analysis of the *Synechocystis* sp. strain PCC 6803 genome indicates that nearly half of the 3,260 genes present are of unknown function (6). In our laboratory we are interested in identifying novel genes required for optimal oxygenic photosynthesis (3, 18). In this study we report the apparent involvement of two genes, *sl10804* and *slr1306*, in the CCM of *Synechocystis* sp. strain PCC 6803.

Two photoautotrophy mutants, 4BA2 and 3ZA12, were previously described (18) as containing single transposon insertions in the *sl10804* and *slr1306* genes, respectively. Both mutants were kanamycin resistant, and neither one exhibited polar effects due to the transposon insertion (18). In our current studies, the kanamycin-resistant wild-type strain K3 was used as a control (13). This strain contains a kanamycin resistance cassette located 5' to the *psbB* gene. Growth conditions were as previously described (18). To test the ability of cells to grow at different CO<sub>2</sub> concentrations, cells of the K3, 4BA2,

and 3ZA12 strains were spotted in a 10-fold dilution series onto petri dishes containing BG-11 medium formulated for either photoautotrophic or photoheterotrophic growth (in 5 mM glucose plus 10 μM DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea]) (18). The plates were incubated for 2 weeks under different CO<sub>2</sub> concentrations at room temperature and a light intensity of ca. 25 μmol m<sup>-2</sup> s<sup>-1</sup>.

For O<sub>2</sub> evolution measurements in the presence of variable amounts of C<sub>i</sub>, the 4BA2, 3ZA12, and K3 cells were grown photoheterotrophically on glucose-containing liquid BG-11 and then transferred to standard BG-11 medium for 2 days and bubbled with either 350 or 50,000 ppm CO<sub>2</sub>. Oxygen evolution rates were measured by polarography after addition of various concentrations of sodium bicarbonate. These experiments were performed with a Hansatech oxygen electrode at a chlorophyll concentration of 10 μg/ml at 24°C and with a light intensity of 800 μmol photons m<sup>-2</sup> s<sup>-1</sup>. Cells were assayed in BG-11 from which Na<sub>2</sub>CO<sub>3</sub> was deleted but with the addition of 20 mM NaCl (2). The assay buffer was bubbled with N<sub>2</sub> gas for more than 2 hours before use. Chlorophyll concentrations were determined using the method of Lichtenthaler (8), while cell numbers were estimated using the equations of Williams (17).

Semiquantitative reverse transcription-PCR (RT-PCR) was performed with the two-step RT-PCR kit (USB Corporation, Cleveland, OH) using primers specific for the *sl10804*, *slr1306*, *ccmN*, *cmpA*, *sbtA*, *psbA*, *rbcL* and *mnpB* genes. The RNase P subunit B gene (*mnpB*) was used as a control for transcript abundance. Total RNA from K3 and the 4BA2 and 3ZA12 mutants grown photoautotrophically at a concentration of 350 or 50,000 ppm CO<sub>2</sub> was isolated using the RiboPure-Bacteria kit (Ambion Inc., Austin, TX).

Figure 1 illustrates the growth of the mutant and wild-type strains under both photoheterotrophic and photoautotrophic conditions at various atmospheric CO<sub>2</sub> concentrations. A 10-fold dilution series is shown (3 × 10<sup>6</sup> to 3 × 10<sup>3</sup> cells per spot). Both mutants grew photoheterotrophically at nearly the same apparent rate as the K3 control strain at all tested CO<sub>2</sub> con-

\* Corresponding author. Mailing address: Department of Biological Sciences, Biochemistry and Molecular Biology Section, Louisiana State University, Baton Rouge, LA 70803. Phone: (225) 578-1555. Fax: (225) 578-7258. E-mail: btbric@lsu.edu.

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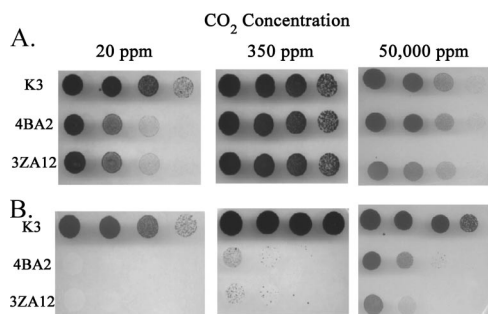


FIG. 1. Growth of the control strain K3 and the 4BA2 and 3ZA12 mutants under photoheterotrophic and photoautotrophic conditions. A. Growth under photoheterotrophic conditions (5 mM glucose plus 10  $\mu$ M DCMU) and various  $\text{CO}_2$  concentrations. B. Growth under photoautotrophic conditions and various  $\text{CO}_2$  concentrations. Dilution series of cells ( $3 \times 10^6$  to  $3 \times 10^3$ ) from the different strains were spotted onto agar plates and grown for two weeks.  $\text{CO}_2$  concentrations are shown above and the individual strains are shown to the left.

centrations (Fig. 1A). In contrast, the mutants were unable to grow photoautotrophically at a  $\text{CO}_2$  concentration of 20 ppm and grew very poorly at 350 ppm, but they grew significantly better at a  $\text{CO}_2$  concentration of 50,000 ppm (Fig. 1B), although not as well as the wild type, which grew well at all tested  $\text{CO}_2$  concentrations. These results indicate that both the 4BA2 and 3ZA12 mutants require high  $\text{CO}_2$  concentrations to grow photoautotrophically. Complementation of both mutants by their respective wild-type genes restored their abilities to grow photoautotrophically at near-wild-type rates with limiting  $\text{CO}_2$  concentrations (data not shown).

Mutant and control cells grown in air or high  $\text{CO}_2$  were assayed for changes in their steady-state photosynthetic  $\text{O}_2$  evolution rates upon the addition of various amounts of  $\text{NaHCO}_3$ . These results are shown in Fig. 2 and summarized in Table 1. The  $V_{\text{max}}$  values of  $\text{O}_2$  evolution appear to be similar for the high- $\text{CO}_2$  and air-grown cells for all strains when results were normalized to the chlorophyll content of the different strains. Interestingly, both mutants contained about 40% less chlorophyll on a per cell basis than did the wild type. The  $K_{0.5}$  values for bicarbonate, however, were significantly different between the high- $\text{CO}_2$  and air-grown cells in all three strains. As expected, the  $K_{0.5}$  value of the K3 strain was  $\sim 10$  times higher in the high- $\text{CO}_2$  cells than in the air-grown cells. This indicated a strong induction of the CCM at low  $\text{CO}_2$  concentrations. The  $K_{0.5}$  values of 4BA2 and 3ZA12, however, were only two to three times higher in the high- $\text{CO}_2$  cells than in the air-grown cells. This indicated that the CCM in the mutant strains was induced poorly in both air-grown mutant cell lines. Additionally, while the  $K_{0.5}$  values of the high- $\text{CO}_2$  cells were very similar between the 4BA2, 3ZA12, and K3 strains, the  $K_{0.5}$  values of the air-grown mutant strains were  $\sim 4$  times higher than the control strain. These differences in  $K_{0.5}$  indicate that both the 4BA2 and 3ZA12 mutants exhibited significantly lower affinities for  $\text{C}_i$  than did the wild type.

RT-PCR was used to evaluate the transcript levels of *sl10804* and *slr1306* at ambient air (350 ppm) and high (50,000 ppm)  $\text{CO}_2$  concentrations. As expected, we were unable to detect the *sl10804* and *slr1306* transcripts in their respective 4BA2 and 3ZA12 mutants (Fig. 3). All three transcripts were detected in

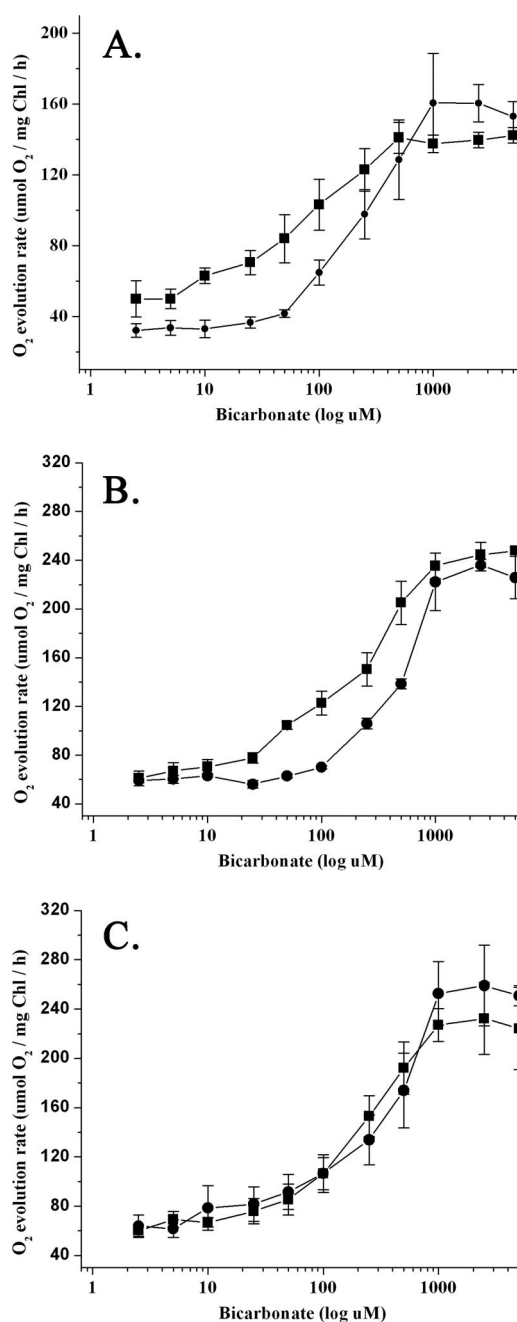


FIG. 2. Photosynthetic  $\text{O}_2$  evolution rates of the wild-type K3 strain and the 4BA2 and 3ZA12 mutants at various concentrations of externally provided bicarbonate. Wild-type strain K3 (A) and the mutant strains 4BA2 (B) and 3ZA12 (C) are shown. The strains were grown photoautotrophically with either a high  $\text{CO}_2$  concentration (50,000 ppm;  $\bullet$ ) or ambient  $\text{CO}_2$  concentration (350 ppm;  $\blacksquare$ ).  $n = 3$  to 5, and error bars show  $\pm 1.0$  standard deviation.

the K3 strain at both high and low  $\text{CO}_2$  concentrations. The level of the *sl10804* transcript in the wild-type strain was 3.6 times higher in the high- $\text{CO}_2$  cells than in the air-grown cells, while there was little difference observed for the *slr1306* transcript with either  $\text{CO}_2$  concentration. Interestingly, we could not detect the *sl10804* transcript in the 3ZA12 mutant cells but did detect the *slr1306* transcript in the 4BA2 mutant cells (Fig.

TABLE 1. Kinetic parameters for  $C_i$  utilization by the control strain K3 and the mutant strains 4BA2 and 3ZA12 grown under ambient air (350 ppm  $CO_2$ ) and high- $CO_2$  (50,000 ppm) conditions<sup>a</sup>

Strain	$V_{max}$ ( $\mu\text{mol/mg chl/h}$ )	$K_{0.5}$ ( $\mu\text{M HCO}_3^-$ )	Chl content ( $\mu\text{g chl}/10^9$ cells)
K3 <sub>air</sub>	142 $\pm$ 4	25 $\pm$ 2	0.104
K3 <sub>CO2</sub>	154 $\pm$ 14	251 $\pm$ 97	ND
4BA2 <sub>air</sub>	248 $\pm$ 3	98 $\pm$ 2	0.063
4BA2 <sub>CO2</sub>	226 $\pm$ 17	272 $\pm$ 12	ND
3ZA12 <sub>air</sub>	232 $\pm$ 24	108 $\pm$ 8	0.066
3ZA12 <sub>CO2</sub>	252 $\pm$ 6	250 $\pm$ 27	ND

<sup>a</sup> The apparent maximal velocity ( $V_{max}$ ) and the bicarbonate concentration which yielded 50% of the apparent maximal oxygen evolution activity ( $K_{0.5}$ ) were determined by examination of the direct plots shown in Fig. 2. The values in the table are means  $\pm$  1.0 standard deviation ( $n = 3$  to 5). Chlorophyll content and cell numbers were determined as described in the text. ND, not determined.

3). The absence of the *sll0804* transcript in the 3ZA12 mutant and the presence of the *slr1306* transcript in the 4BA2 mutant indicates that the Slr1306 protein may act upstream of the Sll0804 component. One hypothesis consistent with this observation is that the Slr1306 protein acts as a positive regulator, either directly or indirectly, of *sll0804* expression.

RT-PCR was also used to examine the expression of a number of other CCM components and control proteins. Interestingly, several other CCM component genes exhibited differential expression in the 4BA2 and 3ZA12 mutants. *CmpA* is a component of the BCT1 high-affinity  $HCO_3^-$  transporter (11), while *SbtA* is a high-affinity,  $Na^+$ -dependent  $HCO_3^-$  transporter (15). Both of these are induced under low- $CO_2$  conditions. We observed this induction pattern in our control strain, K3, with the *cmpA* transcript being strongly upregulated and the *sbtA* transcript being modestly upregulated under low- $CO_2$  conditions. A different pattern was observed in the 4BA2 and 3ZA12 mutants. While *cmpA* was poorly expressed under high- $CO_2$  conditions in the 4BA2 mutant, it was expressed at a high level in the 3ZA12 mutant. Under high- $CO_2$  conditions the *sbtA* transcript also appeared to be expressed at higher levels in both the 4BA2 and 3ZA12 mutants. A similar pattern was observed for the *ccmN* transcript. CcmN is a carboxysome component of unknown function (for a review, see reference 12). In our hands this transcript is moderately upregulated under low- $CO_2$  conditions in the control strain K3. In the 4BA2 and 3ZA12 mutants the *ccmN* transcript is expressed at moderately high levels even under high- $CO_2$  conditions. It should be noted that the control transcripts *psbA* (a membrane protein) and *mpbB* (a cytoplasmic component) do not exhibit differential regulation and are present in equivalent amounts in both mutants and the control cell line under both high- and low- $CO_2$  conditions. These observations further strengthen the hypothesis that the Sll0804 and Slr1306 proteins are putative components of the cyanobacterial CCM.

The *sll0804* gene is predicted to encode a protein of 453 amino acid residues. Using the SMART program (7), three transmembrane domains (amino acids 307 to 329, 349 to 371, and 386 to 408) and a coiled-coil domain (amino acids 268 to 296) were predicted within the protein. In addition, an ATP/GTP-binding domain containing a G1 box (GLVSRGKS), G2 box (T), G3 box (DTPG), and G4 box (KIDL) was located

between amino acids 59 and 211 of the Sll0804 protein. A homology search using the BLAST program (1) revealed that homologues of the Sll0804 protein are present in a wide variety of both  $\alpha$ - and  $\beta$ -cyanobacterial species, including *Cyanothece* sp., *Microcystis aeruginosa*, *Thermosynechococcus* strain BP1, *Synechococcus* strain CC9902, *Prochlorococcus marinus* MIT9313, etc. Within *Synechocystis* sp. strain PCC 6803 several putative paralogues of the Sll0804 protein were identified, including hypothetical proteins encoded by *sll0503* (55% similarity and 38% identity) and *slr1462* (47% similarity and 27% identity).

The *slr1306* gene is predicted to encode a protein containing 485 amino acid residues. The Slr1306 protein was also examined with the SMART program and found to contain a putative N-terminal signal peptide of 37 amino acids and three domains with low compositional complexity (seg domains). An ATP/GTP-binding domain (P loop) was also identified between amino acid residues 128 and 139. BLAST analysis indicated that homologues of the Slr1306 protein were also found in a variety of other cyanobacterial species, including *Cyanothece* sp., *Microcystis*

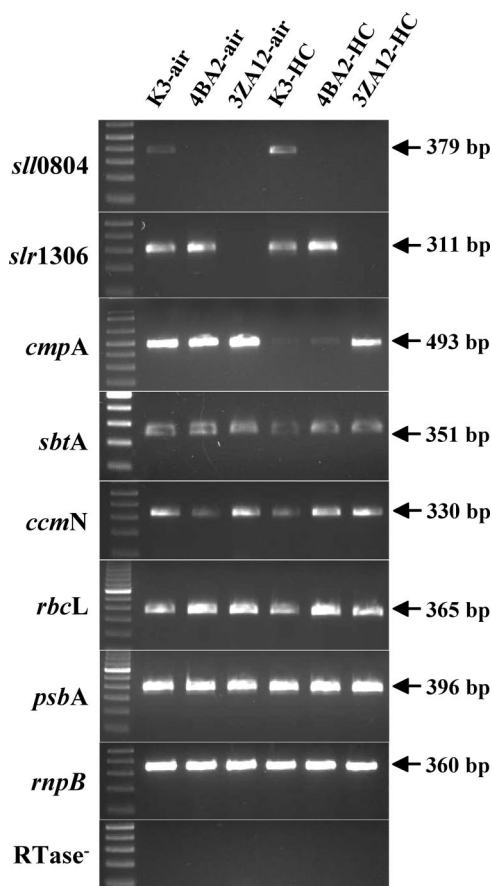


FIG. 3. Semi-quantitative RT-PCR analysis of the total RNA extracted from the wild-type K3 strain and the 4BA2 and 3ZA12 mutants. The strains were grown photoautotrophically under high  $CO_2$  (50,000 ppm; HC) and ambient  $CO_2$  (350 ppm; air) concentrations. Strains and  $CO_2$  concentrations are indicated at the top; the sizes and locations of the PCR fragments are indicated to the right, and the genes analyzed are shown on the left. In the absence of reverse transcriptase (RTase<sup>-</sup>), no bands were observed.

*aeruginosa*, *Synechococcus* sp. strain PCC 7002, *Anabaena* sp. strain PCC 7120, and *Nostoc punctiforme* ATCC 29133. Within *Synechocystis* sp. strain PCC 6803 several putative paralogues of the Slr1306 protein were identified, including hypothetical proteins encoded by slr1462 (47% similarity and 26% identity) and slr0503 (45% similarity and 23% identity).

Growth of many aquatic photosynthetic microorganisms, such as the cyanobacterium *Synechocystis* sp. strain PCC 6803 examined in this study, depends on the activity of a CCM to provide adequate inorganic carbon for photosynthetic carbon fixation (12). Mutations in one or more of the genes involved in the CCM of cyanobacteria often lead to changes of cellular physiology and growth phenotype, such as low growth rates at limiting CO<sub>2</sub> concentrations (9–11, 15, 16). The 4BA2 and 3ZA12 mutants reported in this study lost their abilities to grow photoautotrophically under the low-CO<sub>2</sub> condition, but they regained the ability to grow photoautotrophically under the high-CO<sub>2</sub> conditions. Additionally, the mutants exhibited incomplete induction of the CCM at low CO<sub>2</sub> concentrations, with a consequent decrease in their apparent affinity for C<sub>i</sub>. These phenotypes are the result of single transposon insertions in the slr0804 or slr1306 genes and are complemented by transformation with the respective wild-type genes. These results demonstrate that the slr0804 or slr1306 gene is required for optimal growth of *Synechocystis* sp. strain PCC 6803 at ambient CO<sub>2</sub> concentrations. We hypothesize that these proteins are components of the cyanobacterial CCM.

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