A cyanobacterial AbrB-like protein affects the apparent photosynthetic affinity for CO_2 by modulating low-CO_2-induced gene expression

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Summary

In *Synechocystis* sp. strain PCC 6803, over 450 genes are upregulated following transfer of the cells from a high (1–5% CO_2 in air, HC) to a low level of CO_2 (as in air or lower, LC). This includes *sbtA*, *ndhF3* and *cmpA* involved in inorganic carbon (Ci) uptake. Earlier studies implicated NdhR in the regulation of LC-induced genes but there are indications that additional components are involved. Following extraction of proteins from cells grown under HC and $(NH4)_2SO_4$ fractionation, we have identified LexA and two AbrB-like proteins, Sll0359 and Sll0822, which bind to a fragment of the *sbtA* promoter. Using extracts prepared from LC-grown cells, Sll0822 did not bind to the *sbtA* promoter despite its presence in the cells, suggesting that it may serve as a repressor of LC-induced genes. This is supported by the fact that *sbtA*, *ndhF3* and *cmpA* normally expressed only under LC in the wild-type are transcribed under both HC and LC in a Δ*sll0822* mutant. When grown under HC this mutant exhibits an elevated apparent photosynthetic affinity to Ci, typically observed in the wild-type only under LC. Clearly, expression of genes essential for Ci uptake was sufficient to raise the apparent photosynthetic affinity for external Ci.

Introduction

Induction of the CO_2 concentrating mechanism (CCM) in various cyanobacteria following transfer from a high (1–5% CO_2 in air, HC) to a low level of CO_2 (0.035% CO_2 as in air, or lower, LC) is one example of their ability to acclimate to changing environmental conditions (see Kaplan *et al.*, 1991; Kaplan and Reinhold, 1999; Badger *et al.*, 2002; Giordano *et al.*, 2005; Price *et al.*, 2008 for reviews). The dissolved CO_2 concentration at equilibrium with air is approximately 15-fold lower than the Km(CO_2) of the cyanobacterial ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Marcus *et al.*, 2003). Thus the rate of CO_2 fixation could be seriously limited by the availability of CO_2. Induction of the CCM enables cyanobacteria, such as *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis*), often used as a model organism, to raise the concentration of CO_2 within the carboxysomes in close proximity to Rubisco. Therefore, the cells can overcome the limiting ambient CO_2 concentration in air, inhibit photorespiration (Birmingham *et al.*, 1982; Kaplan and Reinhold, 1999; Giordano *et al.*, 2005; Eisenhut *et al.*, 2006) and grow almost as fast as under high CO_2. Among the genes induced under LC conditions are those engaged in the acquisition of external CO_2 and bicarbonate (Kaplan and Reinhold, 1999; Ohkawa *et al.*, 2000; Shibata *et al.*, 2002; Takahashi *et al.*, 2004; Wang *et al.*, 2004; Nishimura *et al.*, 2008). In *Synechocystis*, three HCO_3^- and two CO_2 uptake systems have been identified (Ogawa and Kaplan, 2003; Price *et al.*, 2008).

The signals and pathways that lead to the induction of LC-specific genes are not fully understood despite significant progress in recent years (Wang *et al.*, 2004; Woodger *et al.*, 2005; Nishimura *et al.*, 2008). Microarray analysis of *Synechocystis* transcripts showed that in addition to genes encoding proteins that function directly in the operation of the CCM, such as those involved in inorganic carbon (Ci) uptake, various genes encoding regulatory elements were also upregulated after CO_2 downshift. These included *slr1214* and *slr1594*, which belong to the PatA subfamily, and three sigma factors: SigD, SigG and SigH (Wang *et al.*, 2004). The LysR type transcription regulator, NdhR (CcmR, Sll1594), was shown to repress several operons containing LC-induced genes, whereas CmpR (Sll0030) acts as an activator of the *cmpA* operon,

Fig. 1. Schematic map of the *sbtA-B* operon. The solid grey area shows the 165 bp fragment, designated p-sbtA, which was used to isolate *sbtA* promoter-binding proteins. The transcription start point (TSP) is indicated. *sbtA* (*slr1512*) and *sbtB* (*slr1513*) are shown, as well as the first ATG of SbtA marked +1. The scheme was not drawn to scale.

encoding the ABC-transporter of HCO_3^-, under LC conditions (Nishimura *et al.*, 2008; Price *et al.*, 2008). However, the fact that both *ndhR* and *cmpR* were upregulated by Ci limitation suggests that the role of NdhR is more complex than just that of a repressor of LC-induced genes (Wang *et al.*, 2004).

The *sbtA* gene (*slr1512*) was chosen as our model system for studying protein–DNA interactions involved in the acclimation of *Synechocystis* from high to low CO_2. It probably encodes a high affinity Na^+/HCO_3^- transporter and is highly induced along with its downstream ORF, SbtB (Shibata *et al.*, 2002; Wang *et al.*, 2004). SbtA is located in the plasma membrane in a complex of approximately 160 kDa, possibly a tetramer (Zhang *et al.*, 2004), and is essential for Na^+-dependent bicarbonate uptake (Shibata *et al.*, 2002) driven by the electrochemical potential of Na^+ (Kaplan *et al.*, 1989). The dependence of HCO_3^- uptake and growth on the presence of Na^+ (Volokita *et al.*, 1984; Espie and Kandasamy, 1992), particularly under alkaline pH conditions, is probably attributable to the $Na \pm HCO_3^-$ nature of SbtA (Kaplan and Reinhold, 1999; Ogawa and Kaplan, 2003).

A fragment of the *sbtA* promoter was used in this study to identify novel regulatory elements involved in its transcription. We focused on three proteins that, in this study, were found to bind to the *sbtA* promoter region: Sll1626, a LexA protein, which has been shown to function as a regulatory element for other *Synechocystis* genes (Domain *et al.*, 2004; Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2005; Patterson-Fortin *et al.*, 2006) and two smaller proteins, Sll0359 and Sll0822, designated CyAbrB (Ishii and Hihara, 2008) that show homology to the AbrB-like family of transcription factors in cyanobacteria. It has recently been reported that Sll0359 interacts with the promoter region of the *hox* operon in *Synechocystis* (Oliveira and Lindblad, 2008) along with LexA (Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2005) and that Sll0822 is involved in the regulation of nitrogen uptake systems, together with NtcA (Ishii and Hihara, 2008). The transcription factor AbrB has been studied extensively in *Bacillus subtilis* where it is involved in the regulation of many genes including those associated with the transition to the stationary phase and in biofilm formation (see Strauch *et al.*, 2007; Chu *et al.*, 2008 and references therein), but the mechanisms determining the specificity of the protein binding to the relevant genes are not known. It is thought that a cradle-loop barrel portion of the *B. subtilis* protein is involved in DNA binding allowing specific recognition of unrelated sequences (Coles *et al.*, 2005). The CyAbrB gene family is highly conserved in cyanobacteria (Ishii and Hihara, 2008; Shalev-Malul *et al.*, 2008) although its specific regulatory functions have not yet been determined.

In addition to the identification of these three proteins that bind to the promoter of *sbtA*, we present evidence that under HC conditions Sll0822 may act as a repressor, or as part of a repressor complex, which is released from the promoter under LC conditions. Studies of the expression of several LC-induced genes in a *sll0822* mutant are consistent with the role of Sll0822 as a repressor. When grown under HC, a mutant where *sll0822* was inactivated showed high apparent photosynthetic affinity close to that observed in LC-grown WT.

Results

Mapping of the sbtA *promoter region*

The significant rise in the abundance of *sbtA* transcript following transfer of the cells from high to low levels of CO_2 (Shibata *et al.*, 2002; Wang *et al.*, 2004), and the fact that *sbtA* is the first gene in an operon containing only two genes (*slr1512* and *slr1513*, see Fig. 1) made it an ideal model for studying promoters that respond to changes in ambient CO_2 concentrations. The transcription start point (TSP) of *sbtA* was identified on RNA isolated from cells grown under low but not HC due to the very low abundance of this RNA in the latter cells (see Shibata *et al.*, 2002 and Fig. 4). Application of modified 5′ RACE techniques (Tillett *et al.*, 2000; Argaman *et al.*, 2001) indicated that the TSP is 168 bp upstream of the ATG with a TATA box located 10–15 bp upstream of the TSP (not shown). The DNA fragment from −137 to −301 (inclusive), designated p-sbtA (Fig. 1), was used in the studies described below.
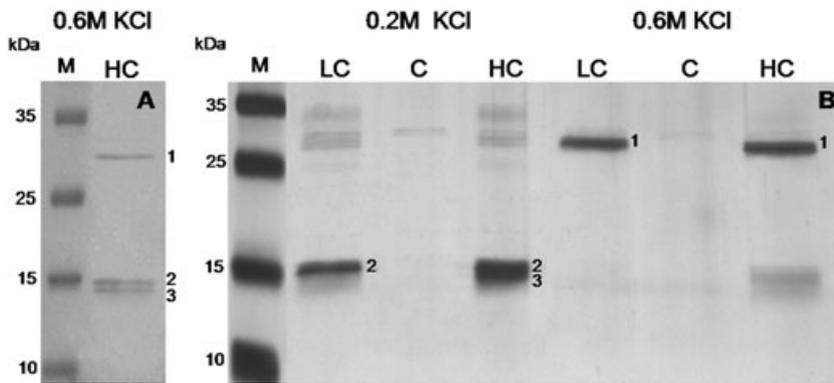
Fig. 2. Proteins bound to p-sbtA. SDS-PAGE of proteins bound to the p-sbtA fragment and eluted with 0.6 M KCl, stained with SeeBand (A) or 0.2 and 0.6 M KCl and silver-stained (B). Proteins were extracted from HC or LC as indicated. Lanes labelled C did not contain any proteins. The size, in kDa, of the molecular markers is shown. Proteins labelled 1, 2 and 3 were identified as LexA, Sll0359 and Sll0822, respectively, by the MS/MS analyses.

Identification of proteins that bind to p-sbtA

To determine which proteins are involved in the transcriptional regulation of *sbtA*, proteins were first extracted from HC-grown *Synechocystis* cells and then fractionated by precipitating with increasing ammonium sulfate concentrations (Koksharova and Wolk, 2002; Shalev-Malul *et al.*, 2008). DIG (digoxigenin)-labelled p-sbtA was used as a probe in gel shift assays to determine which fractions contained proteins that bound specifically to the *sbtA* promoter. The initial results already indicated that under the conditions used here, the proteins that bound most strongly to p-sbtA precipitated between 70% and 90% (w/v) ammonium sulfate (see example in Fig. S1, lane 90). This fraction was therefore used for the DNA binding studies.

TEG (tetra-ethyleneglycol, 15 atom spacer arm)-biotinylated p-sbtA coupled to streptavidin-coated magnetic beads was used as a sequence-specific DNA affinity resin to isolate and identify proteins that bind to *sbtA* promoter in HC-grown cells. After binding to the DNA, the proteins were eluted with 0.6 M KCl, separated by SDS-polyacrylamide gel electrophoresis (PAGE) and stained with SeeBand (Gene Bio Application, Israel). Three major bands were visible on the gel (Fig. 2A) and were identified by MS/MS analyses as LexA (Sll1626), Sll0359 and Sll0822 of approximately 30, 15 and 14.8 kDa respectively. Notably, for an unknown reason, the LexA identified here migrated as a somewhat larger protein than expected in repeated analyses by the MS/MS. An additional very weak protein band, at 32 kDa, was also observed in some of the experiments, but it was not present in sufficient quantity to allow identification by MS/MS.

The effect of CO$_2$ concentration on DNA–protein interactions

To examine a possible role of LexA and the two CyAbrB proteins (Sll0359 and Sll0822) in the induction of genes that are normally expressed under LC conditions, proteins were also extracted from *Synechocystis* cells that were grown under air level of CO$_2$ for 5 days. The proteins from both HC- and LC-grown cells that precipitated in 70–90% (NH4)$_2$SO$_4$ were tested for binding to the biotin-TEG-labelled p-sbtA fragment on a streptavidin magnetic bead resin. The bound proteins were then eluted from the DNA, run on an SDS-polyacrylamide gel and silver-stained (Fig. 2B). Elution of the proteins was done with consecutive steps of 0.2, 0.6 and 1 M KCl but no proteins were observed in the 1 M KCl (not shown). LexA eluted mostly by 0.6 M KCl, but we could not detect significant differences in its level between protein fractions prepared from HC or LC cells.

In general, when proteins extracted from HC cells were used, Sll0359 and Sll0822 were more abundant in the fraction eluted by 0.2 M KCl (Fig. 2B). However, when proteins extracted from LC cells were applied, the smaller protein, Sll0822, was not observed among those eluted from the column (Fig. 2B). The MS/MS analyses confirmed the presence of LexA and Sll0359 but did not detect Sll0822 in the proteins eluted from the DNA. These results are consistent with a possible role of Sll0822 as a repressor that binds to the promoter region of *sbtA* under high but not under LC conditions. The bands observed in the two control lanes (marked C, Fig. 2B), which did not contain any proteins, could be nucleic acids that are also stained by this procedure.

Although Sll0822 was not detected among the proteins bound to p-sbtA *in vitro* after extraction from LC cells (Fig. 2B), Western blot analyses, using an antibody raised against Sll0822, clearly indicated that Sll0822 was present in both HC- and LC-grown *Synechocystis* cells (Fig. 3) in approximately similar amounts. The data also suggested that under the conditions examined here it is mostly found as a dimer. The mechanism that determines the specificity of Sll0822 binding to different promoters and the effect of CO$_2$ concentration on this binding are not known (see *Discussion*, below).
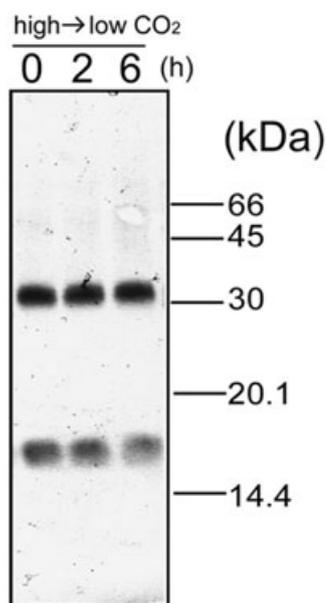
high→low CO$_2$

Fig. 3. Western analysis of proteins extracted from HC- and LC-grown cells. Polyclonal antibodies to His-Sll0822 recombinant protein were used. The proteins were extracted from HC-grown cultures and after 2 and 6 h of exposure to LC. Immunoblotting was performed as described under *Experimental procedures*. The protein extracts were treated with the cross-linker EDC before the SDS treatment.

Expression of LC-induced genes under HC in a Δsll0822 *mutant*

Attempts to isolate a fully segregated Δ*sll0359* mutant were not successful (Ishii and Hihara, 2008; Oliveira and Lindblad, 2008), suggesting that it may be essential for the viability of *Synechocystis*. On the other hand, we were able to isolate a mutant where all the wild-type (WT) copies of *sll0822* were inactivated (Ishii and Hihara, 2008).

We have examined the level of transcripts originating from three different LC-induced genes, *sbtA*, *ndhF3* and *cmpA*, located in different operons (see CyanoBase), as affected by the CO$_2$ concentration experienced by the cells using semi-quantitative (Fig. 4) and quantitative (not
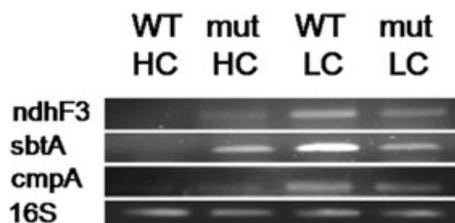

Fig. 4. Semi-quantitative analysis of the abundance of LC-induced genes. The primers used to raise the c-DNA and the RT-PCR for *ndhF3*, *cmpA*, *sbtA* and 16S rDNA are shown in Table 2. No bands were seen in minus controls (data not shown). WT and mut represent the wild-type *Synechocystis* and mutant Δ*sll0822* thereof.

Table 1. The kinetic parameters of photosynthesis with respect to inorganic carbon in *Synecocystis* and mutant Δ*sll0822* thereof as affected by the CO$_2$ concentration during growth.

Strain and growth conditions	K$_{1/2}$ (Ci) μM	V$_{max}$ (μmole O$_2$) (mg chl•h)$^{-1}$
Wild-type, HC	210 ± 5	276 ± 20
Wild-type, LC	25 ± 1	265 ± 19
Mutant, HC	69 ± 5	200 ± 18
Mutant, LC	70 ± 4	187 ± 14

shown) PCR. Using both methodologies, transcripts originating from these genes were hardly detected in WT cells grown under HC but were abundant after transfer of the cells to LC (Fig. 4), in agreement with earlier reports (Ohkawa *et al.*, 1998; Shibata *et al.*, 2002; Wang *et al.*, 2004; Price *et al.*, 2008). In contrast, these genes were significantly expressed in Δ*sll0822* mutant cells maintained under HC level (Fig. 4, and 1.5-, 6-fold in qPCR experiments). Their transcript abundance in the Δ*sll0822* mutant did not increase as much as in the WT cells after transfer from HC to LC conditions (Fig. 4). Microarray analyses performed on RNA isolated from cells grown under LC showed that the expression level of LC-induced genes were lower in the Δ*sll0822* mutant than in WT (Ishii and Hihara, 2008), consistent with the result shown in Fig. 4. Apparently, the mutation alleviated the inhibition of transcription of these genes under HC. This is consistent with the role of Sll0822 as a repressor or as part of a repressor complex which, under HC, binds to the promoters of at least some of the genes induced by LC. The mechanism whereby this protein is dissociated from the promoter following exposure to LC, including the involvement of other components, is not known.

Elevated affinity to external Ci in HC-grown Δsll0822 *mutant*

Data presented in Fig. 4 showed that genes involved in Ci uptake are expressed in the Δ*sll0822* mutant even under HC. Therefore, it was of interest to examine whether these cells show the typical 'LC-characteristics' such as an elevated apparent photosynthetic affinity to external Ci (Kaplan and Reinhold, 1999) even under HC. Analyses of the photosynthetic rate (O$_2$ evolution) as affected by the Ci concentrations showed that this is indeed the case. The apparent K$_{1/2}$(CO$_2$) in HC-grown Δ*sll0822* mutant was closer to that observed in LC-grown WT and significantly lower than in HC-grown WT (Table 1). Clearly, expression of genes essential for Ci uptake led to a remarkable rise in the photosynthetic affinity for external Ci. To the best of our knowledge, this is the first case where a mutant that exhibits high photosynthetic affinity to Ci, regardless of

the CO_2 concentration experienced during growth, was isolated.

Discussion

Identification of novel proteins that bind to p-sbtA

This study focused on the identification of novel transcription factors involved in the cyanobacterial ability to induce LC-dependent genes using the promoter of *sbtA* as a model system. The seminal studies of Burnap and Chauvat and their colleagues (Figge *et al.*, 2001; Wang *et al.*, 2004) implicated NdhR in the regulation of LC-induced genes, including *sbtA*. When grown under HC, the Δ*ndhR* mutant of *Synechocystis* contained a significantly higher level of *sbtA* transcript than did the WT, suggesting that NdhR acts as a repressor of *sbtA* under HC (Wang *et al.*, 2004). However, based on the analyses of various mutants and the response of *Synechocystis* to different environmental conditions, including the presence of glucose (Kahlon *et al.*, 2006), we (and others) have realized that there may be other components involved in the regulation of LC-induced genes, in addition to NdhR. In view of the very clear findings in the earlier studies we did not focus on NdhR but rather on the other three proteins, LexA, and the two CyAbrB proteins shown to bind to the promoter region of *sbtA* (Fig. 2). Nevertheless, it is interesting to note that our MS/MS analyses did not detect NdhR, possibly because of the low abundance of eluted proteins in the expected NdhR size range (about 35 kDa). Furthermore, one of the criteria used to select the p-sbtA fragment for this study was to eliminate three of the four presumptive NdhR binding sites in the region upstream of the 5′-ATG of *sbtA* (Wang *et al.*, 2004). The p-sbtA fragment used here included the TSP identified at −168 bp upstream of the 5′-ATG of *sbtA* (Fig. 1).

The role of Sll0822 and the effect of its inactivation on the photosynthetic performance

The MS/MS analyses indicated that Sll0822 binds to the p-sbtA when proteins extracted from HC-grown cells were used. However, it was missing from the eluted proteins when LC-originated proteins were applied (Fig. 2) although present in the protein extracts (Fig. 3). These data and the observation that *sbtA*, *ndhF3* and *cmpA*, genes that are normally expressed only under LC, were constitutively expressed in the Δ*sll0822* mutant regardless of the CO_2 level, suggested that Sll0822 serves as a repressor of these genes under HC. Its absence in the mutant or removal from LC-induced promoters by some means (see below) alleviated the inhibition and enabled transcription under HC. Notably, the levels of these transcripts were lower than in the LC-induced WT (Fig. 4)

probably reflecting the decline in their level, following the initial rise, during longer acclimation to LC (Eisenhut *et al.*, 2007) as is in fact the case in the Δ*sll0822* mutant grown under HC. The excess energetic cost in mutant Δ*sll0822* under HC due to the operation of Ci transporting systems may explain its slower growth under this condition (Fig. S2). Similarly, the slower growth of the mutant under LC compared with the WT (Fig. S2 and Ishii and Hihara, 2008) may be due to the higher photosynthetic $K1_{/2}(Ci)$ in the mutant (Table 1).

The expression of genes involved in Ci uptake under HC resulted in a remarkable change in the photosynthetic characteristics of these cells (Table 1). The data clearly indicated an elevated affinity to extracellular Ci in mutant Δ*sll0822* grown under HC to levels close to those typically observed only in WT cells grown under LC (Table 1). They also suggested that raising the ability to utilize external Ci sufficed to increase the photosynthetic affinity in *Synechocystis*. This is in agreement with the observation that, in *Synechocystis*, the expression of *ccm* genes involved in the assembly of the carboxysomes, is hardly affected by the ambient CO_2 concentration (McGinn *et al.*, 2003; Wang *et al.*, 2004; Eisenhut *et al.*, 2007). This may not be the case in other cyanobacteria such as *Synecococcus elongatus* PCC 7942 where the abundance of carboxysomes increases significantly during acclimation to LC (see Kaplan *et al.*, 1991; Kaplan and Reinhold, 1999; Giordano *et al.*, 2005; Price *et al.*, 2008). As Sll0822 is also involved in nitrogen uptake (Ishii and Hihara, 2008), it is possible that it helps the cells modulate its metabolism in accordance with the availability of CO_2 and nitrogen sources.

Specificity of the Sll0822 and LexA binding

The inability to isolate a fully segregated Δ*sll0359* mutant and the fact that Δ*sll0822* showed a clear phenotype suggested that these two CyAbrBs play very different roles in *Synechocystis*. In *B. subtilis*, AbrB was implicated in the regulation of close to 100 genes involved in the post-exponential/pre-sporulation period, in the formation of biofilms and other functions (Strauch *et al.*, 1990; Marahiel *et al.*, 1993; Hulett, 1996; Fawcett *et al.*, 2000; Strauch *et al.*, 2007; Chu *et al.*, 2008), but it is not known what determines its specific binding to these genes. Arg-23 and Arg-24, which are located in the centre of the α-helix of the *B. subtilis* protein, are considered crucial for DNA recognition and models have been suggested where the flexible looped-hinge region enables slight conformational changes in the DNA recognition helix, allowing it to bind specifically to unrelated DNA target sequences (Benson *et al.*, 2002; Coles *et al.*, 2005).

In *Synechocystis*, the CyAbrB protein Sll0359 was shown to bind to the promoter of the *hox* operon (Oliveira

and Lindblad, 2008) and to the *sbtA* promoter (Fig. 2). Sll0359 contains the two arginine residues at positions 25 and 26, similar to those present in *B. subtilis* AbrB. This type of CyAbrB protein, designated clade A, bears the sequence LPRREtAK highly conserved in a large variety of cyanobacteria (Ishii and Hihara, 2008). Sll0822, which represents clade B of CyAbrB (Ishii and Hihara, 2008), was shown to bind to p-sbtA (Fig. 2). It is 54% identical to Sll0359 but possesses KE instead of the conserved RR. Interestingly, many of the cyanobacterial CyAbrB proteins possess the highly conserved sequence GDEFEIT/KLG close to the C terminus possibly involved in DNA binding (Coles *et al.*, 2005; Ishii and Hihara, 2008), but the binding specificity is not well understood. In a recent study, we showed that the native AbrB-like protein in *Aphanizomenon ovalisporum* undergoes specific modifications thought to affect its binding specificity and cylindrospermopsin formation (Shalev-Malul *et al.*, 2008). When expressed in *Escherichia coli*, the isolated AbrB from *A. ovalisporum* lacked these modifications and lost its binding specificity (Shalev-Malul *et al.*, 2008).

Post-translational modifications are also expected in Sll0822 because: (i) Sll0822 was present in the total proteins extracted from either LC- or HC-grown cells (Fig. 3) but, when examined under identical conditions, only that from HC binds to p-*sbtA* (Fig. 2B); (ii) when expressed in *E. coli*, the isolated protein lost its specificity and could bind to many different DNA fragments (not shown but see Ishii and Hihara, 2008); and (iii) global analysis of the proteins extracted from *Synechocystis* using 2D-electrophoresis showed two spots for Sll0822 each with a different pI (Sazuka *et al.*, 1999). Nevertheless, we could not detect modifications in either Sll0359 or Sll0822 and it is not known what determines their binding specificity. In the case of Sll0822 the specificity could be mediated via the ratio between the mono and dimeric forms but this ratio did not seem to vary with CO_2 levels (Fig. 3). Naturally, it is possible that Sll0822 interacts with other factors in the cells.

The MS/MS analyses identified LexA among the proteins eluted from the column, in approximately similar amounts when proteins extracted from either HC- or LC-grown cells were used (Fig. 2). LexA was implicated in the regulation of various genes in *Synecocystis* including the *hox* operon, encoding the pentameric bidirectional hydrogenase (Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2005); *crhR*, encoding RNA helicase (Patterson-Fortin *et al.*, 2006); and genes involved in carbon assimilation (Domain *et al.*, 2004). In *E. coli* LexA is involved in the SOS system as a repressor of recombinational DNA repair (see Kelley, 2006; Erill *et al.*, 2007 and references therein), but this is not the case in *Synechocystis* (Domain *et al.*, 2004; Patterson-Fortin *et al.*, 2006). Many LexA binding motifs were recognized in

cyanobacteria, as many as 216 in *Nostoc* sp. PCC 7120 (Sjöholm *et al.*, 2007), but it is not known what determines the specificity of LexA binding to different promoters. Interestingly, we could not recognize LexA binding motifs in the p-sbtA region used here, raising some doubt about the importance of these motifs and their functioning in *Synechocystis*.

The nature of the signal that initiates the induction of the genes involved in the acclimation to LC is still unclear. In a recent study Omata and colleagues demonstrated that presence of 2-phosphoglycolate (2-PG) strongly enhanced the binding of CmpR to the *cmpA* promoter from *Synechococcus elongatus* sp. strain PCC 7942 (Nishimura *et al.*, 2008), supporting the suggestion that 2-PG is involved in the sensing of LC conditions (Kaplan and Reinhold, 1999). This proposal emerged from the fact that shifting the CO_2 concentration from a high to a low level would stimulate the rate of ribulose 1,5-bisphosphate oxygenation by Rubisco and hence the formation of 2-PG and subsequent photorespiration (Eisenhut *et al.*, 2006). It is possible (but not likely) that specificity of Sll0822 to p-*sbtA* is affected by the presence of 2-PG as the differential binding of Sll0822 was observed, *in vitro*, in the absence of 2-PG. Finally, in a set of gel mobility experiments using p-*sbtA* we observed changes in the extent of mobility shifts obtained when combinations of various amounts of the three proteins, LexA, Sll0359 and Sll0822, were used (not shown). This preliminary finding may suggest that the exact level of these proteins (and perhaps also of NdhR) *in vivo*, yet to be determined, and their interaction with other cellular factors plays a critical role in the regulation of LC-dependent genes.

Experimental procedures

Growth conditions

Synechocystis cells were grown as 40 ml cultures in medium BG11 (Stanier *et al.*, 1971) supplemented with 20 mM Hepes-NaOH (pH 7.8) aerated with either 5% CO_2 in air (HC), or with air or 1:1 dilutions of air and CO_2-free air (LC), as indicated. Light intensity was 40 μmol photons m^{-2} s^{-1} provided by cool white fluorescent lamps and the temperature was 30°C. Cultures of mutant $\Delta sll0822$ (Ishii and Hihara, 2008) were supplemented with 25 μg ml^{-1} Kanamycin.

Extraction of proteins and their identification by MS/MS

This was performed essentially as described in Koksharova and Wolk (2002) and Shalev-Malul and colleagues (2008). Cultures of 3–4 l were harvested by centrifugation at 4°C, washed with cold 25 mM Hepes-KOH (pH 7.5) and then re-suspended in buffer A [50 mM Hepes-KOH, pH 7.5, 1 mM disodium EDTA, 2 mM dithiothreitol (DTT), 0.5% Triton X-100, 10% glycerol and a protease inhibitor cocktail (Sigma-

Table 2. The primers used in this study.

Primer	Forward primer (5′→3′)	Reverse primer (5′→3′)	Purpose
sbtP	GGTCACCATCTAGTCCCCAAAAA	GCAGGTTTGAATGCCTATGTCTTG	p-sbtA synthesis
sbtA3		TTCTTCCAACGTAGTGAGGG	5′ RACE
sbtA1	GCTAAACTGCCTAACGTAG	GGCTTGGTAAATATCCCAG	RT-PCR, 5′ RACE
sbtA2	TGGTGCTCTGTATCCCTTTATG	TGCTGAACGATTCCTCAATACT	qPCR, 5′ RACE
ndhF3	TTCCCGCCACTATTCTAAGG	GGTAGGGCAATAAGGGATGC	RT-PCR
16S	AGCGTCCGTAGGTGGTTAT	GTGTCAGTTTCAGCCCAGT	RT-PCR/qPCR
cupA	CCACGCTGTTGATGGATGAT	CCAACTGCACCAACATATCG	qPCR
cmpA	CGAGCGGATTGGGTAGATAA	GCTTCTCGCCACAAATCTTC	RT-PCR

Aldrich, St Louis, MO, USA)]. The cells were then broken with a French press and the lysate centrifuged at 16000 r.p.m. for 20 min at 4°C in a Sorvall SS34 rotor. Ammonium sulfate was added to the supernatant to final concentrations of 20%, 30%, 50%, 70% and 90% (w/v) in an ice-water bath and the precipitates were dissolved in a small volume of buffer A followed by dialysis at 4°C overnight against buffer B (50 mM Hepes-KOH, pH 7.5, 0.1 mM disodium EDTA, 2 mM DTT, 0.1% Triton X-100, 10% glycerol and protease inhibitor cocktail). The dialysed protein fractions were brought to a final concentration of 30% glycerol and stored at −80°C.

DIG-labelling and gel shift

Primers sbtP (Table 2) were used to raise the p-sbtA fragment (Fig. 1). DIG-labelling for the gel shift experiments was done with either the DIG Gel Shift Kit, 2nd Generation (Roche, Mannheim, Germany) or using a 5′ DIG-labelled primer (Metabion, Germany) with similar results. DNA/protein binding conditions were as described in the kit except that a 15 min pre-incubation step with the non-specific DNA was added to the procedure and the incubation period was increased to 30 min. The binding buffer was 20 mM Hepes-NaOH, pH 7.6, 1 mM EDTA, 10 mM $(NH4)_2SO_4$, 1 mM DTT, 0.2% Tween 20 (w/v), 30 mM KCl with 100 ng poly-L-lysine, 100 ng poly (dI-dC) and 200 ng calf thymus DNA added to each 20 μl reaction. Electrophoresis, blotting and chemiluminescence detection were performed as described in Shalev-Malul and colleagues (2008).

DNA affinity chromatography and protein identification

The p-sbt DNA used for the isolation of DNA-binding proteins was prepared using 5′ Biotin-TEG labelled probes (Integrated DNA Technologies, Coralville, Iowa, USA). Binding of the 5′-TEG-biotinylated DNA to magnetic Dynabeads M-280 streptavidin (Dynal, Invitrogen, California, USA) was done according to the manufacturer's instructions. Binding of the proteins that precipitated in 70–90% $(NH4)_2SO_4$ to the DNA was done under similar binding conditions to those used in the gel shift procedure with the following changes: (i) the volume of the reaction was increased to enable the isolation of a larger quantity of protein for analysis and identification; (ii) Pre-incubation of the proteins with non-specific calf thymus DNA and poly (dI-dC) was done for 30 min at room temperature with gentle rotation. The pre-incubated protein solution was then added to the beads with bound DNA and the mixture was gently rotated for 30 min at room tempera-

ture. After four washes with binding buffer to remove unbound proteins, the bound proteins were eluted in small volumes with increasing KCl concentration (0.2 M, 0.6 M and 1 M KCl) in 20 mM Tris (pH 8), 1 mM EDTA (pH 8), 1 mM DTT, 10% glycerol and protease inhibitor. The eluted proteins were analysed by SDS-PAGE (using a gel containing 11% acrylamide) and stained with SeeBand solution (Gene Bio Application, Israel) or silver stain. Bands seen on the gel after SeeBand staining were excised and examined by MS/MS analyses. Identification of proteins bound to p-sbtA was performed using MS/MS as described in Shalev-Malul and colleagues (2008).

RNA extraction

Cells were harvested from 40 ml cultures by centrifugation at 4°C and treated with Tri-Reagent (Molecular Research Center, USA) according to the provided protocol. The RNA pellets were dried at 55°C, dissolved in 20–40 μl DEPC-treated water and then stored at −80°C. DNase (Turbo DNA-free™, Ambion, Austin, TX, USA) was carried out on the RNA samples according to the manufacturer's instructions.

Transcription start point

This was determined by variations of two different 5′ RACE methods described in Tillett and colleagues (2000) and Argaman and colleagues (2001). For the first procedure, the anchor oligonucleotide 5′/phos/CGGACCATTTGGAATTCA CATGCACG/ddC/3′ and reverse anchor primer 5′-CGTGCA TGTGAATTCCAAATGGTCCG-3′ (Integrated DNA Technologies, Coralville, IA, USA) were used along with the *sbtA*-specific primers (Table 2). For the second procedure (Argaman *et al.*, 2001), 2 μg of RNA that was re-suspended in water after DNase treatment, sodium acetate and ethanol precipitation, and 5 units of tobacco acid pyrophosphatase (Epicentre Biotechnologies, Madison, WI, USA) were used for each reaction (total volume 50 μl in buffer supplied by the manufacturer supplemented with 40 units of RiboLock RNase inhibitor (Fermentas, http://www.fermentas.com). The 5′ RNA adaptor (Integrated DNA Technologies, Coralville, IA, USA) used here was the same as that described (Argaman *et al.*, 2001) with the exception of the 5′G. The 5′ RNA adaptor (5 pmole) was ligated to the RNA using T4 RNA ligase (New England Biolabs, USA) for 1 h at 37°C using the buffer supplied by the manufacturer and 40 units of RiboLock. Twenty micrograms of glycogen (Roche, Mannheim, Germany) was added during the procedure as a carrier during each precipi-

tation in sodium acetate and ethanol. The annealing of the primer to the RNA and the reverse transcriptase reaction were done using the Superscript First Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA) and 10 pmol of primer sbtA1 reverse (Table 2) under the conditions described in Tillett and colleagues (2000), except that the 42°C incubation was for one hour rather than 30 min. After incubating at 72°C for 15 min to inactivate the enzyme, samples were taken for PCR using the sbtA2 reverse primer (Table 2) and the adaptor-specific primer 5′-ATGCGCGAATTCCTGTAGAAC-3′. The enzyme used was Taq Zol DNA polymerase (AB Peptides, USA) with buffer supplied by the manufacturer. The PCR products were cloned into the pGEM T-easy vector (Promega, Madison, WI, USA) for transformation into *E. coli* JM109 competent cells (Promega, Madison, WI, USA) and DNA preparations of the plasmids were sequenced.

cDNA synthesis for real-time (qPCR) and semi-quantitative (RT) PCR

For both qPCR and RT-PCR analysis, cDNA syntheses were carried out using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) according to manufacturer's instructions. Five microlitres of diluted cDNA, corresponding to 10 ng total RNA, was used for qPCR. No dilution was made for the RT-PCR amplification. qPCR was carried out on a Rotor-Gene™ 6000 Thermal Cycler (Corbett Research, Australia). Each amplification was carried out in a total volume of 15 µl using the Absolute Blue SYBR Green ROX Mix (Thermo Scientific, ABgene, UK) according to the manufacturer's instructions. Forty cycles of amplification were performed under the following conditions: DNA polymerase activation at 95°C for 15 min, denaturation at 95°C for 10 s, annealing at 56°C for 15 s, product extension at 72°C for 20 s, and signal acquiring at 72°C. PCR reactions were carried out in triplicates. RT-PCR analysis was carried out with the primers indicated in Table 2 on a conventional thermal cycler using 40 cycles of amplification. The PCR products were run on a 0.8% agarose gel in 1× TAE buffer and then stained with ethidium bromide.

Immunoblot analyses

Fifty millilitres of cell cultures at the exponential growth phase was harvested by centrifugation. The pellet was re-suspended in 200 µl of breakage buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl). The cell suspension was mixed with approximately 100 µl volume of glass beads (diameter 0.1 mm; BioSpec Products) and cells were disrupted (four cycles of vigorous agitation for 2 min each followed by cooling on ice for 1 min). After removal of unbroken cells and debris by centrifugation at 700 g for 3 min, cell extracts were mixed with an equal volume of 2× SDS sample buffer [62.5 mM Tris-HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 0.01% BPB (w/v)]. SDS-PAGE was performed with gels containing 15% acrylamide. After blotting to a PVDF membrane (Immobilon-P; Millipore), samples were probed with polyclonal antibodies to His-Sll0822 recombinant protein. The bound antibodies were detected with goat anti-rabbit IgG secondary antibodies conjugated to alkaline phosphatase. To see the oligomeric state of Sll0822, cell extracts were treated with a cross-linker, 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, hydrochloride (EDC), before the addition of 2× SDS sample buffer. Samples were incubated with 25 mM EDC, 10 mM Tris-HCl (pH 8.0), 200 mM NaCl and 20% glycerol (v/v) at 25°C for 2 h.

Photosynthetic measurements

The rate of CO_2-dependent O_2 evolution as a function of C_i concentration was determined using a Clark type O_2 electrode (PS2108, Passport dissolved O_2 sensor Roseville, CA, USA) essentially as described in Kaplan and colleagues (1988). The cells were harvested by centrifugation and re-suspended in a CO_2-free medium containing 10 mM NaCl and 20 mM Hepes-NaOH, pH 7.8. They were then placed in the O_2 electrode chamber at 30°C, 300 µmol photons m^{-2} s^{-1} and allowed to utilize the C_i in their medium until they reached the CO_2 compensation point. Aliquots of NaHCO$_3$ of known concentrations were injected to raise the C_i concentration by known increments while measuring the resulting rise in the rate of O_2 concentration in the chamber.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. An example of a gel shift to identify the relevant protein fraction. DIG-labelled p-sbtA was used as a probe. Proteins were isolated from HC-grown *Synechocystis* and fractionated by salting out with ammonium sulfate. Lane C: no proteins added, showing the DIG-labelled probe. Lanes 30, 70, 50, 90: proteins from 30%, 70%, 50% and 90% (w/v) saturated ammonium sulfate fractions respectively. Note that the order of the loading to these lanes was deliberately switched to reduce false positives due to spillover. Conditions for DNA-protein binding are as described in *Experimental procedures*.

Fig. S2. Growth of *Synechocystis* (WT) and the mutant Δ*sll0822* (Mut) thereof. The effected of the CO_2 concentration during growth was examined. The cells were grown as described in *Experimental procedures*, HC was 5% CO_2 in air and LC was a dilution of 1:1 air and CO_2-free air.

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