A cyanobacterial AbrB-like protein affects the apparent photosynthetic affinity for CO₂ by modulating low-CO₂-induced gene expression

Judy Lieman-Hurwitz,1 Maya Haimovich,1 Gali Shalev-Malui,1 Ai Ishii,2 Yukako Hihara,2 Ariel Gaathon,3 Mario Lebendiker4 and Aaron Kaplan1*
1Department of Plant and Environmental Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel.
2Department of Biochemistry and Molecular Biology, Saitama University, Saitama 338-8570, Japan.
3Bletterman Laboratory, Interdepartmental Equipment Unit, Hebrew University of Jerusalem, Faculty of Medicine, Jerusalem 91120 Israel.
4The Wolfson Centre, Hebrew University of Jerusalem, Jerusalem 91904, Israel.

Introduction

Induction of the CO₂ concentrating mechanism (CCM) in various cyanobacteria following transfer from a high (1–5% CO₂ in air, HC) to a low level of CO₂ (0.035% CO₂ 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 CO₂ concentration at equilibrium with air is approximately 15-fold lower than the Km(CO₂) of the cyanobacterial ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Marcus et al., 2003). Thus the rate of CO₂ fixation could be seriously limited by the availability of CO₂. 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 CO₂ within the carboxysomes in close proximity to Rubisco. Therefore, the cells can overcome the limiting ambient CO₂ 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 CO₂. Among the genes induced under LC conditions are those engaged in the acquisition of external CO₂ 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 HCO₃⁻ and two CO₂ 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 CO₂ downshift. These included slr1214 and slr1594, which belong to the PatA subfamily, and the 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.
encoding the ABC-transporter of HCO\textsubscript{3}\textsuperscript{−}, 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 (srl1512) was chosen as our model system for studying protein–DNA interactions involved in the acclimation of Synechocystis from high to low CO\textsubscript{2}. It probably encodes a high affinity Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{−} 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\textsuperscript{+}-dependent bicarbonate uptake (Shibata et al., 2002) driven by the electrochemical potential of Na\textsuperscript{+} (Kaplan et al., 1989). The dependence of HCO\textsubscript{3}\textsuperscript{−} uptake and growth on the presence of Na\textsuperscript{+} (Volokita et al., 1984; Espie and Kandasamy, 1992), particularly under alkaline pH conditions, is probably attributable to the Na\textsuperscript{±}/HCO\textsubscript{3}\textsuperscript{−} 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\textsubscript{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 (srl1512 and srl1513, see Fig. 1) made it an ideal model for studying promoters that respond to changes in ambient CO\textsubscript{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.
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 CO2 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 CO2 for 5 days. The proteins from both HC- and LC-grown cells that precipitated in 70–90% (NH4)2SO4 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 CO2 concentration on this binding are not known (see Discussion, below).
Expression of LC-induced genes under HC in a Dsll0822 mutant

Attempts to isolate a fully segregated Dsll0359 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 CO2 concentration experienced by the cells using semi-quantitative (Fig. 4) and quantitative (not 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 Dsll0822 mutant cells maintained under HC level (Fig. 4, and 1.5-, 6-fold in qPCR experiments). Their transcript abundance in the Dsll0822 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 Dsll0822 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 Dsll0822 mutant

Data presented in Fig. 4 showed that genes involved in Ci uptake are expressed in the Dsll0822 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 (O2 evolution) as affected by the Ci concentrations showed that this is indeed the case. The apparent K1/2(CO2) in HC-grown Dsll0822 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

<table>
<thead>
<tr>
<th>Strain and growth conditions</th>
<th>K1/2 (Ci) µM</th>
<th>Vmax (µmole O2) (mg chl·h)−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type, HC</td>
<td>210 ± 5</td>
<td>276 ± 20</td>
</tr>
<tr>
<td>Wild-type, LC</td>
<td>25 ± 1</td>
<td>265 ± 19</td>
</tr>
<tr>
<td>Mutant, HC</td>
<td>69 ± 5</td>
<td>200 ± 18</td>
</tr>
<tr>
<td>Mutant, LC</td>
<td>70 ± 4</td>
<td>187 ± 14</td>
</tr>
</tbody>
</table>

Table 1. The kinetic parameters of photosynthesis with respect to inorganic carbon in Synechocystis and mutant Dsll0822 thereof as affected by the CO2 concentration during growth.
the CO₂ 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 ΔndhR 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 Δsll0822 mutant regardless of the CO₂ 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 Δsll0822 mutant grown under HC. The excess energetic cost in mutant Δsll0822 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 Δsll0822 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 CO₂ concentration (McGinn et al., 2003; Wang et al., 2004; Eisenhut et al., 2007). This may not be the case in other cyanobacteria such as Syneococcus 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 CO₂ and nitrogen sources.

Specificity of the SiI0822 and LexA binding

The inability to isolate a fully segregated Δsll0359 mutant and the fact that Δsll0822 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 2008).
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 LPRREIAK 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 cytidospermospin 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 CO2 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 Synechocystis including the hox operon, encoding the pentameric bidirectional hydrogenase (Gutekunst et al., 2005; Oliveira and Lindblad, 2005); orhR, 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 CO2 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% CO2 in air (HC), or with air or 1:1 dilutions of air and CO2-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 Δsll0822 (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-
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-labled 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 Heps-NaOH, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₄SO₄, 1 mM DTT, 0.2% Tween 20 (w/v), 30 mM KCl with 100 ng poly-c-lysine, 100 ng poly (dl-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, IA, 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)₂SO₄ 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 (dl-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 temperature. 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 cocktail. The eluted protein fractions were brought to a final concentration of 30% glycerol and stored at –80°C.

**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/C GGACCATTTGGAATTCA CATGCAGCAGGACGAGAGA CTTAGTGCCCAATATGTTCC-3′ (Integrated DNA Technologies, Coralville, IA, USA) was 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 (Epipenic 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-
tion 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′-ATGCCGGAATTCTCTAGAAG-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 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 1x 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 2x 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 2x 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 CO₂-dependent O₂ evolution as a function of C₃ concentration was determined using a Clark type O₂ electrode (PS2108, Passport dissolved O₂ sensor Roseville, CA, USA) essentially as described in Kaplan and colleagues (1988). The cells were harvested by centrifugation and re-suspended in a CO₂-free medium containing 10 mM NaCl and 20 mM Hepes-NaOH, pH 7.8. They were then placed in the O₂ electrode chamber at 30°C, 300 μmol photons m⁻² s⁻¹ and allowed to utilize the C₃ in their medium until they reached the CO₂ compensation point. Aliquots of NaHCO₃ of known concentrations were injected to raise the C₃ concentration by known increments while measuring the resulting rise in the rate of O₂ concentration in the chamber.

Acknowledgements

Funding for this work was obtained from the Israeli Science Foundation (ISF), German-Israeli Science Foundation (GIF) for Scientific Research and Development, the German DFG and the German BMBF with the Israeli MOST.

References


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-stbA 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 Δsll0822 (Mut) thereof. The effect of the CO₂ concentration during growth was examined. The cells were grown as described in Experimental procedures, HC was 5% CO₂ in air and LC was a dilution of 1:1 air and CO₂-free air.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.