A novel transcriptional regulator, Sll1130, negatively regulates heat-responsive genes in *Synechocystis* sp. PCC6803

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A conserved hypothetical protein, Sll1130, is a novel transcription factor that regulates the expression of major heat-responsive genes in *Synechocystis* sp. PCC6803. *Synechocystis* exhibited an increased thermostolerance due to disruption of *sll1130*. Δ*sll1130* cells recovered much faster than wild-type cells after they were subjected to heat shock (50°C) for 30 min followed by recovery at 34°C for 48 h. In Δ*sll1130* cultures, 70% of the cells were viable compared with the wild-type culture in which only 30% of the cells were viable. DNA microarray analysis revealed that in Δ*sll1130*, expression of the heat-responsive genes such as *htpG*, *hspA*, *isiA*, *isiB* and several hypothetical genes were up-regulated. Sll1130 binds to a conserved inverted-repeat (GGCGATCGCC) located in the upstream region of the above genes. In addition, both the transcript and protein levels of *sll1130* were immediately down-regulated upon shift of wild-type cells from 34 to 42°C. Collectively, the results of the present study suggest that Sll1130 is a heat-responsive transcriptional regulator that represses the expression of certain heat-inducible genes at optimum growth temperatures. Upon heat shock, a quick drop in the Sll1130 levels leads to de-repression of the heat-shock genes and subsequent thermal acclimation. On the basis of the findings of the present study, we present a model which describes the heat-shock response involving Sll1130.

Key words: cis-regulatory element, DNA microarray, heat acclimation, Sll1130, transcription factor.

INTRODUCTION

Micro-organisms acclimatize to heat by rapid up-regulation of a specific set of genes that encode HSPs (heat-shock proteins) [1,2]. During heat acclimation, the HSPs act as molecular chaperones or proteases and facilitate refolding of heat-denatured proteins, stabilization of protein structure, solubilization of aggregated proteins and degradation of irreversibly damaged proteins [1–3].

In the cyanobacterium *Synechocystis* sp. PCC6803 (hereafter known as *Synechocystis*), expression of heat-shock genes such as *hspA*, *htpG*, *groES*, *groEL*, *dnaK*, *dnaJ* and *clpB* are up-regulated immediately after the cells are shifted to high temperature [4,5]. Inactivation of heat-responsive genes such as *htpG*, *hspA*, *dnaK2* (molecular chaperones), *isiA* and *isiB* (iron- and heat-regulated proteins), *sigB* and *sigC* (alternative sigma factors) result in thermal sensitivity in *Synechocystis*, indicating their physiological importance during heat stress [6–11]. In *Synechocystis* the small HSP, HspA, has been reported to be an amphitropic protein that stabilizes heat-stressed membranes and binds denatured proteins for subsequent chaperone-mediated refolding [8]. *Synechocystis* becomes sensitive to high temperature on disruption of *hspA* [9,12]. Inactivation of *htpG*, another important heat-shock gene, causes *Synechocystis* to become relatively more sensitive to high temperature than caused by inactivation of the same gene in *Escherichia coli*, *Bacillus subtilis* or *Aggregatibacter actinomycetemcomitans* [9,13–15], indicating its significance in photosynthetic organisms. Insertional inactivation of *isiA*, which was originally reported to be up-regulated under iron-limiting conditions, caused a heat-sensitive phenotype in *Synechocystis* [11]. Collectively, the literature therefore suggests that accumulation of major HSPs seems to be essentially required for thermal tolerance in *Synechocystis* [16]. *Synechocystis* there are two-component signal-transduction systems involving a sensory histidine kinase and a cognate response regulator that transduce the environmental signals to the target genes and act independently of transcription factors [17–20]. It has been reported that a sensory kinase, Hik34 (histidine kinase 34), is involved in the regulation of several heat-responsive genes and the Δ*hik34* deletion mutant showed increased thermal tolerance [4]. In addition to the signal transduction involving Hik34, positive regulation by alternative sigma factors, SigB and SigC, was suggested to activate the transcription of *hspA* and *groEL* genes in *Synechocystis* [11,21]. In various Gram-negative and Gram-positive bacterial systems, different negative-control mechanisms regulate heat-shock genes [22]. In *Streptomyces albus* HAIR (HspR-associated inverted repeats)/HspR controls the dnaK operon and clpB gene [23]; in *B. subtilis* CtsR negatively regulates clpC and clpP genes [24], and the CIRCE/HrcA system negatively regulates *grpE–dnaK–dnaJ* and/or *groE* operons [25,26]. However regulation of important heat-shock genes is poorly understood in cyanobacteria.

A hypothetical protein, Sll1130, was previously reported to be associated with PSII (photosystem II) in *Synechocystis* [27]. Since it was reported to be associated with PSII, we were curious as to its role in PSII structure and function. However, we found that this is not a PSII-associated protein, instead it is a novel heat-responsive transcriptional regulator. This protein negatively regulates expression of *hspA*, *htpG*, *isiA*, *isiB* and several genes that encode hypothetical proteins in *Synechocystis* by binding to the cis-regulatory element located upstream of these genes.
demonstrate that mutation in sll1130 leads to enhanced thermal tolerance in Synechocystis.

EXPERIMENTAL

Bacterial strains and culture conditions

Synechocystis, a glucose-tolerant strain that was originally obtained from Dr J. G. K. Williams (Dupont de Nemours, Wilmington, DE, U.S.A.), served as the wild-type. Wild-type cells were grown photoautotrophically at 34°C in BG-11 medium [28] buffered with 20 mM Hepes/NaOH (pH 7.5) under continuous illumination at 70 μmol of photons/m² per s as described previously [29]. The Δsll1130 culture, in which the sll1130 gene was disrupted by inserting a Kan^R (kanamycin-resistance gene) cassette, was grown under the same conditions as described above with the exception that the culture medium contained kanamycin at 25 μg/ml in precultures. Growth of the culture was monitored by measuring absorbance at 730 nm using a spectrophotometer (Shimadzu UV-160A). For heat treatment, wild-type culture was grown to mid-exponential phase (an absorbance of ~0.7 at 730 nm) at 34°C and then shifted to a water bath maintained at 42°C with continuous illumination (70 μmol of photons/m² per s). Cells were harvested before and after heat treatment, and total cellular proteins and total RNA were isolated. For heat-shock and recovery experiments, 50 ml of wild-type and Δsll1130 mutant cultures were grown at 34°C to mid-exponential phase and then incubated at 50°C for 30 min in a water bath. The heat-shocked culture tubes were shifted back to 34°C and allowed to recover from the heat shock. Cells were collected before heat shock and after 48 h of recovery for viability tests.

Generation of the Δsll1130 mutant

We generated a Δsll1130 mutant of Synechocystis by insertional inactivation of the sll1130 gene. A DNA fragment containing the sll1130 ORF (open reading frame) with 69 bp upstream and 197 bp downstream flanking regions was amplified by PCR with the following primers: sll1130-F (5'-TATGGCTGCCACCGCCGACACTATGAC-3') and sll1130-R (5'-GGTCCAGCGATCTAGTTATCTTTCCAG-3'). The PCR-amplified 614 bp fragment was ligated to a linear T-vector (GeNei™ INSTANT Cloning kit; catalogue number Ge80000, GeNei™) according to the manufacturer's instructions (catalogue number 68400, GeNei™). The resulting plasmid pT-sll1130 was used to inactivate the sll1130 ORF by performing an in vitro transposon reaction according to the protocol provided by the manufacturer (EZ::Tn5™ <KAN-2> Insertion kit; catalogue number EZ1982K, Epicentre). The plasmid DNA construct in which the sll1130 ORF was disrupted with the Kan^R cassette was designated as pTssl1130::kan^R. This construct was used to transform Synechocystis cells. The site of insertion of the kanamycin cassette was located by sequencing the pTssl1130::kan^R construct using the Kan-RP1 primer. Genomic DNA of the Δsll1130 mutant cells grown for several rounds in BG11 medium was prepared and the extent of the replacement of wild-type copies of sll1130 with the sll1130::kan^R was checked using primers sll1130-F and sll1130-R. The mutant thus generated was named Δsll1130.

Viability test

Synechocystis wild-type and Δsll1130 cells were stained with ViaGram™ Red^+ Bacterial Gram Stain and Viability kit according to the manufacturer’s instructions (catalogue number V-7023, Molecular Probes, Invitrogen). SYTOX Green stain was diluted with water (3 μl to 60 μl). To 50 μl of cell suspension, 2.5 μl of the diluted SYTOX Green was added and incubated at room temperature (28°C) for 15 min. Then 10 μl of the stained cell suspension was examined with a confocal microscope (TCSSP-2, AOBS 4 channel UV and visible; Leica).

Purification of Sll1130 and antibody generation

Antibodies against Sll1130 were raised in rabbits with His-tagged Sll1130 of Synechocystis, which had been overexpressed in E. coli as described previously [30]. The sll1130 ORF was PCR-amplified with the following primers: sll1130-ExF (5'-GGCGCACATGAGCACATACAGAACAATTGG-3') and sll1130-ExR (5'-GGCGAAGCGTTACCGAGTTTAAACACATGGGG-3'). The NcoI and HindIII restriction sites are underlined. The ninth nucleotide G was substituted for A to create an NcoI site in the forward primer. The amplified ORF of sll1130 was inserted into pET-28a(+) at the NcoI and HindIII sites to generate pET-sll1130. The C-terminally His-tagged Sll1130 protein was expressed in BL21(DE3)pLysS, which had been transformed with pET-sll1130 and was purified using HIS-Select™ Nickel-Affinity gel (catalogue number P6611, Sigma) according to the manufacturer’s instructions. The purified Sll1130 protein was examined under non-denaturing (i.e. the protein was mixed with loading dye without 2-mercaptoethanol and SDS and was not boiled before electrophoresis; the running buffer did not contain SDS), denaturing (the protein was mixed with loading dye containing 2-mercaptoethanol and SDS and was boiled before electrophoresis) and semi-denaturing (the protein was mixed with loading dye containing SDS, without 2-mercaptoethanol and was not boiled before electrophoresis) conditions to determine the oligomeric state of the Sll1130 protein. The purified protein was used to generate anti-Sll1130 antibody in New Zealand white rabbits with Freund’s complete adjuvant (primary) and Freund’s incomplete adjuvant (booster). Serum was collected after the second booster, diluted 3000-fold in 3% BSA and used for the detection of Sll1130 protein by Western blot Analysis. Animal experiments were carried out under the appropriate Institutional guidelines.

Gel-permeation chromatography of Sll1130

Gel-permeation chromatography of purified Sll1130 protein (100 μg) was performed on a Sephacryl S-100 column (dimensions 50 cm × 0.5 cm) in 50 mM phosphate buffer (pH 7.0). The gel-filtration molecular mass markers BSA and CA (carbonic anhydrase) were dissolved at a concentration of 0.1 mg/ml in 100 μl of purified Sll1130 protein solution (catalogue numbers A8654 and C5024, Sigma–Aldrich) and loaded on to the chromatography column (100 μl of sample on a Sephacryl S-100, GE Healthcare). Approximately 37 fractions (each equivalent to 0.25 ml) were collected for analysis. Eluates were estimated for the presence of proteins by UV absorption at 280 nm. Equal volumes of collected fractions were resolved by SDS/PAGE (15% gel) and subsequently the amount of protein present in each band was estimated densitometrically. The Coomassie-Blue-stained gels were scanned in a Gel Logic 212 PRO (Kodak) and the intensity of each protein band corresponding to molecular mass markers and Sll1130 were further calculated by Carestream Molecular Imaging software (Carestream Health).

Western blotting analysis

Western blotting analysis was performed as described previously with some modifications [30]. Polyclonal antibodies raised in
rabbit against His–Sll1130 protein were used as the primary antibody, and alkaline-phosphatase-linked antibody raised in goat against rabbit IgG was used as the secondary antibody. After SDS/PAGE separation, proteins were blotted on to PVDF membrane (Immobilon-P; Millipore) in a semi-dry transfer apparatus (TE77-PWR semi-dry transfer unit, GE Healthcare). Levels of Sll1130 were determined immunologically with the NBT (Nitro Blue Tetrazolium)/BCIP (5-bromo-4-chloroindol-3-yl phosphate) chromogenic detection system according to the manufacturer’s instructions (Sigmafast BCIP/NBT; catalogue number B5655, Sigma–Aldrich).

Gel mobility-shift assays

A 283 bp DNA fragment containing the region upstream from a translation start site of slr1788 was obtained by PCR using Cy3 (indocarboxyanine)-labelled primers 5′-GAGAAACTGATCT-TGAGAGATGG-3′ and 5′-GATTTTGGTAATTTGATCAT-GGCC-3′. Cy3-labelled primers were obtained from Eurofins Genomics. The Cy3-labelled PCR fragment was incubated with various concentrations of His–Sll1130 in 25 μl of binding buffer [10 mM Tris/HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA, 2% glycerol, 0.5 mM DTT (dithiothreitol), 1 mM MgCl2 and 4 μg of poly(dI/dC)] for 30 min at room temperature. The mixtures were subjected to electrophoresis on a native 6% polyacrylamide gel at 60 V. A specific competitor DNA was prepared by annealing the following oligonucleotides: 5′-TGCCCCAAGAAGATGGC-GATCGCGGTTGGATCCGA-3′ and 5′-GCTCCGAATC-CACAGGCGGATCGGCATTTCGTTGGGCA-3′. The inverted repeat, which was predicted to be the binding site of Sll1130, is underlined in the oligonucleotides. Analyses of gel images were carried out using epiluminescence and a Cy3 filter in a Syngene G:box image analyser.

Preparation of cDNA for DNA microarray analysis

Wild-type Synechocystis cells and Δsll1130 mutant cells that were grown at 70 μmol of photons/m2 per s (50 ml) were killed instantaneously by the addition of 50 ml of ice-cold 5% (w/v) phenol in ethanol, and then total RNA was extracted as described previously [31]. The RNA was treated with DNase I (Nippon Gene) to remove contaminating DNA. cDNAs, labelled with fluorescent dyes [Cy3 and Cy5 (indodicarbocyanine); Amersham Pharmacia Biotech], were prepared from 10 μg of total RNA with an RNA fluorescence labelling core kit [MMLV (Moloney murine leukaemia virus), version 2.0 Takara] according to the manufacturer’s instructions.

DNA microarray analysis

 Genome-wide analysis of transcript levels was performed with DNA microarrays as described previously [30]. We used a Synechocystis DNA microarray (CyanoCHIP, Takara) containing 3079 of the 3168 ORFs (97% of all genes, with the exception of transposon-related genes) of the Synechocystis genome for finding genes with altered expression due to mutation in slr1130.

qRT-PCR (quantitative real-time PCR)

RNA isolated from wild-type and Δsll1130 cells before and after heat treatment was used for cDNA synthesis with the Affinity Script cDNA synthesis kit, according to the manufacturer’s protocol (catalogue number 600559, Agilent). qRT-PCR was carried out using the Power SYBR Green Master Mix kit (Applied Biosystems). Each reaction was carried out in a 25 μl volume containing 12.5 μl of Power SYBR Green Master Mix, 0.2 μM of each primer and 5 μl of diluted cDNA (35 ng). All reactions were run in duplicate using a qRT-PCR instrument (Mx3000P, Agilent). The instrument was programmed for 95 °C for 10 min, and then 40 cycles of 30 s at 95°C, 60 s at 60°C and 60 s at 72 °C. For each reaction the melting curve was analysed and the PCR product was run on an agarose gel in order to confirm the specificity of the RT (reverse transcription)–PCR. Expression levels were normalized using the gapl gene as an internal reference. Primers used for qRT-PCR are listed in Supplementary Table S1 (at http://www.biochemj.org/bj/449/bj4490751add.htm).

Northern blot analysis

Total RNA was extracted from cells, and Northern blotting analysis was performed as described by Los et al. [31]. DNA fragments corresponding to the sll1130 and rpmB genes were conjugated with alkaline phosphatase (Alkphos Direct kit; Amersham Pharmacia Biotech) and the resultant conjugates were used as probes. After hybridization the blots were soaked with CDP-Star solution (Amersham Pharmacia Biotech) and signals from hybridized mRNAs were detected with a luminescence image analyser (LAS-4000, Fuji).

Identification of a common cis-regulatory element upstream of genes up-regulated by mutation of sll1130

A 500 bp upstream and/or intergenic DNA region of each gene whose expression was up-regulated due to mutation in slr1130 was submitted to MEME (motif extraction by multiple expectation maximization) version 4.3.0. MEME was run using the default parameters. MEME identified a common inverted repeat (cis-regulatory element) upstream of the majority of the genes whose expressions were up-regulated by the mutation. A consensus nucleotide sequence was generated by aligning the cis-regulatory elements upstream of all the up-regulated genes (Figure 7B).

RESULTS

Sll1130 is a conserved protein with an unknown function

The ORF of sll1130 encodes an unknown protein in Synechocystis [32]. Figure 1 shows the alignment of the top ten BlastP hits of Sll1130 protein. The protein is highly conserved and its orthologues are present in bacteria of different taxonomic phyla such as Proteobacteria, Cyanobacteria and Spirochaetes. Secondary structure prediction of the Sll1130 protein by GORIV suggests that the protein has four helices spread throughout the length (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_gor4.html). The first three helical regions were well conserved among all orthologues of Sll1130 (Figure 1). The protein has a putative conserved pemK superfamily domain. Proteins containing the pemK domain are common to many bacterial species and are reported to play roles in stress-mediated growth regulation or plasmid maintenance [33]. Some members of the PemK superfamily of proteins are known to be toxin/antitoxin proteins that regulate cell growth under antibiotic or abiotic stress [34]. However, Sll1130 from Synechocystis exhibited neither toxic properties nor was it involved in plasmid maintenance (results not shown). Since the Sll1130 protein is well conserved among various bacterial species and showed similarity to the pemK superfamily of proteins, it is
Figure 1  Multiple sequence alignment of the Sll1130 from Synechocystis sp. PCC 6803 and its homologues retrieved from the NCBI database

The sequences were aligned using the ClustalW algorithm. Identical amino acids are shown in black and conserved amino acid substitutions are in grey. The first three helices (H), starting from the N-terminal end of Sll1130 are conserved among all orthologues of Sll1130. Helix regions were predicted using the GOR IV secondary structure prediction tool. The PemK domain on Sll1130 protein was identified using a Pfam database search and is indicated as a grey bar. Sll1130, Synechocystis sp. PCC 6803; YP_001265356.1, Sphingomonas wittichii RW1; CAM75735.1, Magnetospirillum gryphiswaldense MSR-1; ZP_08928879.1, Thioalkalivibrio thiocyanoxidans ARh4; NP_840995.1, Nitrosomonas europaea ATCC 19718; YP_115395.1, Methylococcus capsulatus str. Bath; CCE25566.1, Methylomicrobium alcaliphilum; CBX31194.1, Desulfobacterium sp.; PCC8801_3647, Cyanothece sp. PCC8801; ZP_01386989.1, Chlorobium ferrooxidans DSM 13031. Likely that it may have an important physiological function and play role(s) in survival of the cell.

Mutagenesis of sll1130

In an attempt to ascertain the function of the Sll1130 protein, sll1130 was inactivated, as shown in the schematic diagram in Figure 2(A), generating the Δsll1130 mutant. In this mutant all copies of the sll1130 gene were replaced by the disrupted copies of sll1130 genes as confirmed by the fact that when genomic DNA of wild-type cells was used as a template with specific primers (sll1130-F and sll1130-R), a PCR product of 614 bp corresponding to the sll1130 ORF and its upstream and downstream flanking regions was amplified (Figure 2B). In contrast, when the genomic DNA of Δsll1130 cells was used as the template with the same set of primers, a 1814 bp DNA fragment corresponding to the wild-type fragment (614 bp) plus the inserted kanamycin gene (1200 bp) was amplified (Figure 2B).

Δsll1130 mutant cells exhibited increased thermal tolerance

Both wild-type and Δsll1130 mutant cells of Synechocystis exhibited similar growth profiles at 34°C despite the complete inactivation of sll1130 gene, in the Δsll1130 mutant (Figure 3). But when exponentially growing cells of wild-type and Δsll1130 mutant strains were heat-treated at 50°C for 30 min and then allowed to recover at 34°C, it was observed that Δsll1130 mutant cells were relatively more tolerant to heat than wild-type cells (Figure 4). We measured cell survival after a high-temperature treatment by taking small aliquots of cell suspension and counting the viable and non-viable cells under confocal microscopy after staining them with SYTOX Green fluorescent dye. When examined under a fluorescence microscope, cyanobacterial cells appear as bright red cells due to auto-fluorescence emitted from chlorophyll a. Dead cells appeared as green fluorescent cells due to entry of SYTOX Green dye into the cell [35]. Before heat shock, both wild-type and Δsll1130 cells appeared as bright red fluorescent cells, indicating that the cells were equally viable at the optimal growth temperature, 34°C (Figures 4A and 4B). Following heat shock at 50°C for 30 min, cells were allowed to recover at 34°C for 48 h and viability was measured again. While only 30% of the total wild-type cells were viable following this heat shock (cells emitting red fluorescence over green fluorescence) (Figures 4C and 4E), 70% of the Δsll1130 cells remained viable (Figures 4D and 4E).
that disruption of sll1130 leads to increased thermostolerance of Synechocystis cells.

**Sll1130 protein is a tetramer**

Sll1130 was expressed and purified as a His–Sll1130 protein from *E. coli*. We determined the oligomeric state of Sll1130 by fractionating the purified native Sll1130 protein along with known appropriate markers, such as BSA (66 kDa) and CA (29 kDa) on a gel-exclusion column. Protein quantification by densitometric analysis of the eluates indicated that BSA and CA eluted with maximum intensity in fraction numbers 15 and 19 respectively (Figure 5A). We observed that the native Sll1130 protein eluted with maximum intensity in fraction number 15 along with BSA. The estimated molecular mass of native Sll1130 was four times higher than that calculated on the basis of its amino acid sequence, suggesting Sll1130 in its native form exists as a tetramer. In addition, on native-PAGE, Sll1130 protein appeared as a single protein band above the 43 kDa marker protein (ovalbumin) (Figure 5B). However, the pure His–Sll1130 protein resolved as a single protein band of 14.6 kDa on SDS/PAGE, implying that the molecular mass of its monomeric form is 12.9 kDa after subtracting 1.7 kDa accounted for by the His-tag (Figure 5C). Interestingly, under semi-denaturing conditions (in the absence of 2-mercaptoethanol), in addition to the protein band that was expected at 14.6 kDa, another protein band at approximately 30 kDa was observed. This suggests the involvement of a disulfide bridge between two monomers in dimer formation, facilitated by a cysteine residue that can promote the formation of a disulfide bridge. Incubation of purified Sll1130 with SDS resulted in dissociation of tetramers to dimers, as indicated by the anti-Sll1130 antibody reacted with two monomers by non-covalent interactions. Figure 5(D), shows the immunodetection of Sll1130 in the soluble (S) and insoluble (I) membrane fractions extracted from wild-type and Δsll1130 Synechocystis cells; Sll1130, purified His–Sll1130 was detected using an anti-Sll1130 antibody.
soluble nor insoluble fractions of Δsll1130, suggesting absence of SII130 functional protein in the mutant (Figure 5D).

Identification of target genes of SII130

It is likely that SII130, which we identified as an oligomeric protein with conserved helices, may serve as a transcriptional regulator in Synechocystis. We therefore sought to identify the target genes of SII130. We performed DNA microarray analysis of wild-type and Δsll1130 mutant cells grown under optimal growth conditions (70 μmol of photons/m² per s; 34°C). Table 1 shows the list of genes whose expression levels were significantly affected by the disruption of sll1130. Genes whose mean induction (Δsll1130/wild-type) was greater than 2.0 were considered to be up-regulated, whereas those with an induction less than 0.5 are listed as down-regulated (Table 1). It is of note that following inactivation of sll1130 the expression of certain heat-responsive genes, such as hspA, htpG, isiA and isiB, was up-regulated along with several other genes that encode hypothetical proteins (Table 1). Four genes, ssl2245, sll1130, cysS (cysteinyl-tRNA synthetase) and codA (cysteine deaminase) were down-regulated due to mutation in sll1130. Both the ssl2245 gene, which is located upstream of the sll1130 ORF, and the codA gene, which is located downstream of the sll1130 ORF (http://www.kazusa.or.jp/cyanobase/Synechocystis/genes/sll1130), were strongly down-regulated due to insertional inactivation of sll1130. These observations suggest that there is a common regulatory mechanism which controls the expression of these two genes. Observed down-regulation of cysS, codA and sll1130 genes is consistent with the DNA microarray expression data generated upon heat shock in Synechocystis [4].

qRT-PCR analysis confirms DNA microarray expression changes

Differences in the expression of five genes, namely sll1788, isiA, frpC, hspA and ssl2245, observed in the sll1130 mutant compared with the wild-type were further confirmed by qRT-PCR analysis (Figure 6A and Table 1). These results suggest that SII1130 acts as a transcriptional negative regulator of hspA, htpG, isiA, isiB, frpC and several other hypothetical genes, and therefore these genes may be up-regulated by de-repression in Δsll1130 cells. Genes coding for HspA and HtpG are known to play important roles in heat acclimation and the survival of Synechocystis cells at high temperature [6,7,9,12,36]. isiA and isiB have been reported to be up-regulated under heat and iron-limiting conditions and play important roles in protection from heat-stress conditions [11].

Heat-induced expression of the hspA gene was studied both in wild-type cells and the Δsll1130 mutant by qRT-PCR to ascertain reasons for the heat-tolerant phenotype (Figure 6B). The temporal expression of the hspA gene for a period of 180 min indicated an initial increase in expression by 10 min and a gradual decline by 180 min in both the wild-type and Δsll1130

Table 1 Effect of mutation in sll1130 on genome-wide expression of genes

Wild-type and Δsll1130 cells were grown at 34°C for 16 h. Each value indicates the ratio of levels of mRNA from Δsll1130 mutant cells to wild-type cells. The values shown are the means ± range for two independent experiments. The numbering of the ORF corresponds to that described in Kaneko et al. [32]. The complete list of gene expression in the wild-type and Δsll1130 mutant can be accessed at http://www.kazusa.or.jp/kegg-bin/get HText?htext = Exp_DB&hier = 1.

<table>
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<th>Gene</th>
<th>Product</th>
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<tr>
<td>ssl1788</td>
<td>Hypothetical protein</td>
<td>8.3 ± 1.0</td>
</tr>
<tr>
<td>ssl1789</td>
<td>Hypothetical protein</td>
<td>6.3 ± 0.6</td>
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<td>Two-component response regulator (PatA subfamily)</td>
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<td>Hypothetical protein</td>
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<td>16.6 kDa small heat-shock protein, molecular chaperone</td>
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</tr>
<tr>
<td>ssl1920</td>
<td>Type 4 pilin-like protein</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>ssl0248</td>
<td>Flavodoxin</td>
<td>2.2 ± 0.9</td>
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<tr>
<td>ssl1780</td>
<td>Transposase gene of IS4 family insertion sequence</td>
<td>2.2 ± 0.0</td>
</tr>
<tr>
<td>ssl0870</td>
<td>Hypothetical protein</td>
<td>2.1 ± 0.0</td>
</tr>
<tr>
<td>ssl0430</td>
<td>Hypothetical protein</td>
<td>2.1 ± 0.0</td>
</tr>
<tr>
<td>ssl1149</td>
<td>Salt-enhanced periplasmic protein</td>
<td>2.1 ± 0.2</td>
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<tr>
<td>ssl2245</td>
<td>Hypothetical protein</td>
<td>0.3 ± 0.15</td>
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<tr>
<td>ssl1130</td>
<td>Hypothetical protein</td>
<td>0.2 ± 0.04</td>
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<tr>
<td>ssl0158</td>
<td>Cysteinyl-tRNA synthetase</td>
<td>0.1 ± 0.04</td>
</tr>
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<td>ssl1237</td>
<td>Cysteine deaminase</td>
<td>0.1 ± 0.01</td>
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Sll1130, a heat-responsive transcriptional repressor

Figure 6 Changes in the expression levels of selected genes due to mutation in sll1130

(A) Confirmation of DNA microarray results by qRT-PCR. slr1788, frpC, isiA, and hspA showed a higher level of expression in the Δsll1130 mutant than wild-type, as measured by microarray (open bars) and by qRT-PCR (solid bars). Similar results were obtained in two independent experiments; data are presented as means ± S.D. (B) hspA mRNA levels during the course of heat treatment in the wild-type and Δsll1130 cells. Both wild-type (○) and Δsll1130 (○) cells were grown at 34°C for 16 h and then subjected to heat stress (42°C) for 10, 60, 120 and 180 min. The fold change of hspA mRNA levels in heat-treated wild-type and Δsll1130 cells was expressed relative to its levels in WT-34°C cells (means ± S.D. for three independent experiments). WT, wild-type.

mutant cells. However, in the mutant cells hspA expression was significantly greater than in the wild-type cells all through the heat-treatment period (Figure 6B). Relatively higher levels of hspA mRNA in Δsll1130 mutant cells compared with wild-type cells explain the heat-tolerant phenotype of the Δsll1130 mutant. Thus collectively the results of the present study, together with the available literature [6,7,9,11,12,36], suggest that the enhanced thermostolerance of the Δsll1130 strain could be due to elevated transcript and corresponding protein levels of these important heat-stress-inducible genes in Synechocystis (Table 1, and Figures 4 and 6).

A common cis-regulatory element was detected upstream of genes up-regulated in Δsll1130

If Sll1130 is a negative regulator of transcription of all of the genes that were up-regulated by its inactivation, then a common cis-regulatory element that is recognized by the Sll1130 protein would be expected upstream of these genes. As predicted, we found a common inverted-repeat sequence, GGCGATCGCC, upstream

Figure 7 Presence of a conserved inverted repeat in the upstream region of genes that are up-regulated due to mutation in sll1130

Upstream DNA region of genes that are induced due to mutation in sll1130 were submitted to MEME (http://meme.sdsc.edu/meme/cgi-bin/meme.cgi) for prediction of the conserved DNA element. (A) Shows the alignment of an inverted repeat in the upstream region of genes up-regulated due to mutation in sll1130. (B) Logo representation of the conserved regulatory element. (C) Location of the inverted repeat in the upstream region of genes up-regulated due to mutation in sll1130. Open arrows in the schematic representation are coding regions of up-regulated genes. Straight line, length of upstream DNA region; rectangles, inverted repeat.
of most of the genes whose expression was up-regulated due to mutation in sll1130 (Figures 7A and 7B). This common inverted repeat may be the target binding site of Sll1130 and probably acts as a cis-regulatory element. We predicted this inverted repeat computationally using MEME software as described in the Materials and methods section. In some of these up-regulated genes the identified DNA element GGCGATCGCC is located at two places in the upstream region, similar to that of the LacI repressor site [37] (Figure 7C).

Specific binding of Sll1130 to the cis-regulatory element located upstream of slr1788

The binding of the cis-regulatory element located upstream of slr1788 with Sll1130 protein was studied using a gel mobility-shift assay (Table 1). As shown in Figure 8, purified His-Sll1130 protein retarded the electrophoretic mobility of the upstream DNA fragment of slr1788, and this shift was observed to be concentration-dependent (Figure 8). We confirmed that the observed retardation was due to binding of Sll1130 protein with the upstream fragment of slr1788 by adding 100-fold excess of double-stranded oligonucleotides (40 bp) covering the computationally predicted cis-regulatory element, which completely eliminated the retarded fragment (Figure 8). Taken together with the presence of the same cis-regulatory element upstream of the other genes up-regulated by inactivation of sll1130, these data clearly implicate Sll1130 as a negative regulator of these genes.

Expression of the sll1130 gene is down-regulated upon an upward shift in temperature

DNA microarray analysis has revealed that expression of the sll1130 gene is down-regulated upon shift of wild-type Synechocystis cells to a high temperature [4]. We observed down-regulation in the sll1130 expression in Synechocystis cells grown at 34°C and then shifted to 42°C. Significant levels of sll1130 mRNA were detected before exposure to 42°C, indicating that the sll1130 gene is constitutively expressed (Figure 9A). Within 10 min of an upward shift in temperature to 42°C, the level of sll1130 transcript decreased by approximately 80% and remained at a similar level up to 7 h after the shift (Figures 9A and 9C).

Western blot analysis with an anti-Sll1130 antibody also indicated that the levels of Sll1130 protein decreased significantly when Synechocystis cells grown at 34°C for 16 h were shifted to 42°C (Figures 9B and 9C). The anti-Sll1130 antibody detected Sll1130 at a molecular mass of approximately 15 kDa (Figure 9B). The level of Sll1130 protein decreased to 60% within 30 min of incubation at 42°C and reached a minimum of 50% of the original level within 180 min, suggesting that Synechocystis cells down-regulate the expression of the sll1130 gene and reduced its protein upon heat treatment (Figure 9C). This is in agreement with our previous DNA microarray study in which there was down-regulation of sll1130 upon a 20 min heat shock at 42°C [4]. These observations indicated that Synechocystis cells down-regulated the expression of the sll1130 gene and corresponding Sll1130 protein upon an upward shift in temperature.

DISCUSSION

The major HSPs that have been so far well characterized in terms of their function are GroES, GroEL, DnaJ, DnaK, HspA, HtpG and several proteases. Understanding the regulation of the heat-shock genes is essential to unravelling the molecular basis of heat acclimation. In bacteria, negative regulation of several heat-shock genes, such as dnaK, dnaJ, groES, groEL, grp, clpB, clpC and clpP, has been previously reported [23–26]. However, to the best of our knowledge, a repressor involved in negative regulation of hspA and htpG has not so far been reported. The present paper is the first report on a conserved hypothetical protein, Sll1130,
The sll1130 transcript and protein levels upon an upward shift in temperature (Figure 10B). The absence of the Sll1130 protein in the sll1130 mutant cells resulted in elevated levels of heat-responsive proteins that conferred increased thermostability.

**AUTHOR CONTRIBUTION**

Pilla Sankara Krishna performed the experiments, analysed the data and wrote the paper. M. Karthik Mohan and Balaga Radha Rani generated the sll1130 mutant. Iwane Suzuki helped perform the DNA microarray analysis. Sisnithy Sivajivi contributed to writing the paper and in discussions. Jogadhenu Prakash defined the project strategy, supervised all aspects of the work and wrote the paper.

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**REFERENCES**


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SUPPLEMENTARY ONLINE DATA

A novel transcriptional regulator, Sll1130, negatively regulates heat-responsive genes in Synechocystis sp. PCC6803

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Table S1  Primers used for qRT-PCR

<table>
<thead>
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<th>Gene</th>
<th>Forward primer (5′→3′)</th>
<th>Reverse primer (5′→3′)</th>
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<tr>
<td>slr1788</td>
<td>TTCGACTTCGTTACATCCTG</td>
<td>CGTTAAGCAGATCGTGGT</td>
</tr>
<tr>
<td>sll1009 (frpC)</td>
<td>ATGTTGGCGATGATACCGTC</td>
<td>CATTGCCGTCGCCACCATA</td>
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<tr>
<td>sll1514 (hspA)</td>
<td>GAAACTGAAGAAGCCTATGTG</td>
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<tr>
<td>sll1247 (isiA)</td>
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<td>CCAGGAACAGGAGATGATGA</td>
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<td>CCGTAGAAGTTATTGTGTTAGT</td>
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<tr>
<td>sll0884 (gap1)</td>
<td>ACCATCTCAACTACAACGCC</td>
<td>GGTCATCAATCCTCCACAAT</td>
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