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ABSTRACT

EXPRESSION OF HEAT SHOCK GENES *HSP16.6* AND *HTPG*

IN THE CYANOBACTERIUM, *SYNECHOCYSTIS* SP. PCC 6803

by Feng Fang

Heat shock proteins (HSP) are found in all living organisms in response to elevated temperatures, and protect cells from heat damage. In this dissertation, *hsp16.6* and *htpG* were studied in the cyanobacterium, *Synechocystis* sp. PCC 6803. The *hsp16.6* transcriptional start point was positioned 44 base pairs (bp) upstream of ATG translation start codon. A reporter vector was constructed by ligating the 265 bp upstream fragment onto the upstream region of the *lacZ* coding sequence. β-galactosidase analysis indicated the 265 bp region did not induce *lacZ* gene expression in *E. coli*; although *lacZ* expression was induced when the *Synechocystis groESL* promoter was used. In *Synechocystis* cells, *lacZ* was expressed when the 265 bp fragment was used as a promoter. Cold stress and ethanol did not induce *lacZ* expression, while heat shock, salt stress, sorbitol, hydrogen peroxide and high light induced *lacZ*. Deletions of the 265 bp region demonstrated that the induction of β-galactosidase activity was lost when a region upstream of the transcriptional start point was deleted.

The *htpG* null mutant was obtained by inserting a chloramphenicol resistance cassette (Cm^r) in the *htpG* coding sequence. The *htpG* null mutant (Δ*htpG*), *Δhsp16.6,* and the double mutant, *ΔhtpG::hsp16.6* cells grew well at 30°C and 37°C, but not at 40°C. This suggests that HtpG and HSP16.6 proteins do not have an essential role at normal and mildly elevated temperatures. Cell growth, cell survival rate, and oxygen electrode measurements demonstrated that *ΔhtpG, Δhsp16.6,* and *ΔhtpG::hsp16.6* cells

were sensitive to heat stress. Decreased basal and acquired thermotolerance were observed when mutants were heat shocked, with *ΔhtpG::hsp16.6* being the most sensitive. A comparison of mutants showed that *Δhsp16.6* was more sensitive to heat shock than *ΔhtpG*.

In summary, a 265 bp region upstream of *hsp16.6* was demonstrated to response to heat shock, salt stress, sorbitol, hydrogen peroxide and high light, and the deletion of a region upstream of the transcriptional start point resulted in the loss of heat induction. *HtpG* was demonstrated to be essential during heat stress, and the deletion of *htpG* caused decreased basal and acquired thermotolerance.

Keywords: Heat shock, cyanobacteria, *Synechocystis*, *htpG*, *hsp16.6*, regulation, reporter, promoter, basal thermotolerance, acquired thermotolerance.

EXPRESSION OF HEAT SHOCK GENES *HSP16.6* AND *HTPG* IN THE CYANOBACTERIUM, *SYNECHOCYSTIS* SP. PCC 6803

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Table of abbreviations

bp	base pair(s)
CIRCE	controlling inverted repeat of chaperone expression
Clp	caseinolytic protease
Cm^r	chloramphenicol resistance cassette
cpn60	chaperonin 60
CtsR	class three stress gene repressor
Cyt C	cytochrome c
HAIR	HspR associated inverted repeat
HrcA	heat regulation at CIRCE
HSP	heat shock protein
HtpG	high temperature protein G
IbpB	inclusion body associated protein B
IR	inverted repeat
Kan^r	kanamycin resistance cassette
kD	kilodalton
LB media	Luria-Bertani media
LMW	low molecular weight
ORF	open reading frame
P265	265 bp upstream region of *hsp16.6* coding sequence
PBS buffer	phosphate buffered saline buffer
PCC	Pasteur Culture Collection
RT-PCR	Reverse transcription-polymerase chain reaction
ROSE	repression of heat shock gene expression
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sig	sigma factor
Smhsp	small heat shock protein

DEDICATION

I dedicate this dissertation to my wife, Wang Wei and son, David Fang.

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Chapter 1 Introduction

Heat Shock in Cyanobacteria

The heat shock response is an adaptation found in all living organisms. At normal growth temperatures, heat shock proteins (HSPs) are present in low concentrations, however, during heat stress their concentration increases dramatically [19, 33, 55, 56]. A group of proteins are expressed transiently in response to elevated temperatures, and the other proteins are reduced [42, 56]. HSPs are divided into five conserved classes according to size: HSP100, HSP90, HSP70, HSP60 and small HSP (smHSP) or low molecular weight (LMW) HSPs (below 30 kD) [19, 56, 66, 74]. HSPs have been shown to play important roles in the protection of organisms under heat stress [19, 56]. They protect cells from heat damage by binding to other polypeptides for transport to other regions of the cell or across membranes, stabilizing existing and nascent protein structures, renaturing unfolded or aggregated polypeptides, and degrading some potentially hazardous polypeptides [19, 33, 56, 72, 76].

The phenomenon of the heat shock response was first described in *Drosophila melanogaster* in 1962. To date, some 200 papers have been published on the heat shock response in cyanobacteria. In summary, there are five factors that may affect thermotolerance in cyanobacteria (cell growth temperature, pretreatment at the elevated temperature, composition of the cellular membrane, constitutive protein, and heat shock proteins). Studies have shown that by acclimating to a higher growth temperature, cyanobacteria increased thermotolerance to even higher temperature [27, 54]; Pretreatment at a sub-lethal temperature resulted in acquired thermotolerance (induced or

developed thermotolerance) by inducing a wide range of heat shock proteins which were detected by methods such as SDS-PAGE, 2-D gel, Northern hybridization, Western blot, and DNA chip analysis [3, 29, 46, 49, 59]. All five major classes of HSPs have been cloned and characterized from cyanobacteria [13, 38, 40, 49, 68, 59]. Mutagenesis studies have shown that some HSPs directly influence thermotolerance [37, 46, 49, 68], and like HSPs from other organisms, HSPs may work together as a network to protect cells from heat stress, and to protect membranes *in vitro* [69, 71]. Evidence suggests that HSP expression in cyanobacteria may be controlled by a different regulatory mechanism than *E. coli* [62].

The study of HSPs in cyanobacteria may help us understand the heat stress phenomenon in chloroplasts of higher plant. Cyanobacteria have a simple, prokaryotic structure, and grow rapidly. *Synechocystis* PCC 6803 is naturally competent for DNA transformation, so it is convenient for molecular manipulation [36]. In addition, the genome sequence of *Synechocystis* PCC 6803 was completed in 1996 (http://www.kazusa.or.jp/cyano/cyano.html). Four other, *Thermoynechococcus elongatus* BP-1, *Anabaena* sp. PCC 7120, *Gloeobacter violaceus* PCC 7421, and *Chlorobium tepidum* TLS genome sequences have been completed (http://www.kazusa.or.jp/cyano /cyano.html). The cyanobacteral genome projects will most certainly be helpful for heat shock research.

Thermotolerance and acclimation to high growth temperatures in cyanobacteria

When organisms are pretreated at a sublethal temperature, they can survive at a higher temperature that would normally be lethal. This is called acquired

thermotolerance. The ability to tolerate elevated temperatures without pretreatment is defined as basal thermotolerance [22, 56]. When *Anabaena* sp. PCC 7120, *Plectonema boryanum* PCC 6306, and *Synechococcus* sp. PCC 7942 were pretreated at sub-lethal temperatures, the pretreatment increased cell survival at an elevated previously lethal temperature [3]. This phenomenon also was observed in other cyanobacteria [12, 68]. In addition, acclimation to high growth temperatures also confers thermotolerance in cyanobacteria. For *Synechocystis* sp. PCC 6803 cells, the optimal growth temperature is 30°C; however, when cells were cultured from 22°C to 36°C, the increased growth temperature protected cells at previously-lethal temperatures [39]. *Synechocystis* cells grown at 35°C (acclimation temperature) could tolerate higher temperature treatment than cells grown at 25°C. Acclimation of 35°C growth temperature increased the oxygen evolution rate, the viability of cells, PSII reaction center activity, and the permeability of plasma membranes after heat shock [27]. The effect of acclimation to the higher growth temperature was also studied in *Anacystis nidulans* [64]. Low-temperature-growth cells (LTG, 25°C) were more sensitive to heat stress than high-temperature-growth cells (HTG, 38°C). The pigment absorption spectra showed that LTG cells were damaged under heat stress. This suggests that less heat susceptible sites in the HTG cells may result in increased thermotolerance [64]. Proteins play an important role in acclimation to the higher temperature [54]. Protein PsbU is an extrinsic protein in the photosystem II complex in cyanobacteral cells. In *Synechococcus* sp. PCC 7002 cells, *psbU* gene knockout decreased photosynthesis system thermal stability when cells acclimate at moderately high temperatures [54]. The *psbU* gene null mutation also decreased cellular thermotolerance to high temperature [54]. In *Synechocystis* sp. PCC 6803 cells, the

acclimation to higher growth temperature increased the expression of *dnaK*, *groESL*, *cpn60*, and *hsp17* (also called *hsp16.6* or *hspA*), and membrane fluidity, and thus, may affect thermotolerance [23].

Membranes and thermotolerance

Studies have shown that higher temperature treatment changes the membrane state, and thus, affect thermotolerance [23]. In *Synechocystis* PCC 6803, higher incubation temperature for 9 hours increased the saturated fatty acid content of total lipids in cells [11]. Mutation of fatty acid desaturases resulted in the elimination of dienoic lipid molecules (but not trienoic lipid molecules), and also decreased thermotolerance of photosynthetic oxygen evolution [20]. When *Synechocystis* cells acclimate to higher growth temperature (35°C), the permeability of plasma membranes increased [27]. Treatment of *Synechococcus vulcanus* at 72°C results in leakage of low-molecular-weight substances through cell membranes, and thus, may decrease thermotolerance [26].

Thermotolerance is not only affected by the membrane state itself, but also by the interaction between membrane and heat shock proteins. Cellular membranes have been considered a part of a signal pathway to activate heat shock genes, so cells can survive during heat stress [18, 23]. In *Synechocystis* PCC 6803, HSP16.6 was shown to integrate into the membrane. *In vitro* analysis showed that the protein-lipid integration suggested a preference for the liquid crystalline phase and maintained physical order in model lipid membranes [71]. Besides HSP16.6, GroEL was also found to associate with thylakoid membranes [31]. Mutation of *hsp16.6* decreased the membrane integrity during heat

stress [37]. Some proteins other than HSPs may also have a protective role during heat stress by integration into the membrane [54]. In *Synechococcus* sp. PCC 7002, proteins such as PsbU, an extrinsic protein of photosystem II, protected PS II against heat-damage [53]. Cyt c-550 also has a similar role in protecting membranes against heat damage [52].

Heat shock genes and functional analysis

In response to elevated temperatures, a group of proteins are expressed transiently to protect cells from thermal damage [56]. In cyanobacteria, a wide range of heat inducible proteins have been found. An early study using SDS-PAGE showed that heat shock decreased *Synechococcus* sp. PCC 6301 cell growth, and at the same time, a set of heat induced proteins were found when cells were heat shocked at 47°C [4]. In *Anabaena* sp. PCC 7120, *Plectonema boryanum* PCC 6306, and *Synechococcus* sp. PCC 7942, when cells were pretreated at a sub-lethal temperature, 2-D gels revealed 33 heat shock proteins in *Anabaena*, 35 proteins in *Plectonema boryanum*, and 19 proteins in *Synechococcus* [3]. Similar results were obtained in *Synechocystis* PCC 6803 cells. Heat shock proteins were produced in cells cultured at 22°C and transformed at 36°C [39]. Besides heat shock, some other stresses such as UV light and nalidixic acid could also induce a set of HSPs in the cyanobacterium *Phormidium laminosum* [48]. In the cyanobacterium, *Synechococcus* sp. PCC 7942, *Synechocystis* sp. PCC 6803, and *Synechococcus* sp. PCC 7418, general heat shock proteins were found after salt stress, heat shock, and light stress [15].

All five major classes of HSPs have been characterized from cyanobacteria [13, 36, 38, 40, 49, 59, 68]. Two major chaperones, the HSP60 prokaryotic homolog, GroEL,

and the HSP70 prokaryotic homolog, DnaK have been the most studied in cyanobacteria. Three other classes of HSPs, the HSP100 prokaryotic homolog, ClpB, the HSP90 prokaryotic homolog, HtpG (high temperature protein), and small HSPs (smHSP) are considered minor chaperones [70]. Due to our current limited understanding of cyanobacteral genetics, heat shock gene characterization has been studied in only six cyanobacteral species, *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942, *Aphanothece halophytica* [35, 65]*, Thermosynechococcus elongatus, Anabaena* sp. L-31 [59] and *Synechococcus vulcanus*. The first heat shock genes isolated, *groEL* and *dnaK,* were cloned and characterized from *Synechocystis* sp. PCC 6803 [5]. Thus far, *clpB* (*hsp100*) from *Synechococcus* sp. PCC 7942 [7, 12, 13], htpG (hsp90) from *Synechocystis* sp. PCC 6803 [14], and *Synechococcus* sp. PCC 7942 [68], *dnaK* (*hsp70*) from *Aphanothece halophytica* [35, 65], *Synechocystis* sp. PCC 6803 [5, 71], *Synechococcus* sp. PCC 7942 [50, 51], *groEL* (*hsp60*) from *Synechocystis* sp. PCC 6803 [17, 31, 32, 40, 71], *Synechococcus* sp. PCC 7942 [6, 8, 49], *Anabaena* sp. L-31 [59], *Synechococcus vulcanus* [16], *smhsp*, *hspA* from *Synechococcus vulcanus* [61, 62] and *Thermosynechococcus elongatus* [30], *hsp16.6* from *Synechocystis* sp. PCC 6803 [38], and *orf7.5* from *Synechococcus* sp. PCC 7942 [46] were identified to be induced by heat shock. Isolated genes from cyanobacteria are listed in Table 1.

Several cyanobacteral heat shock genes have been mutated to help elucidate their function. Inactivation of *Synechococcus clpB* (*hsp90*) did not affect growth under optimal growth conditions. During heat stress, the *clpB* null mutant lost thermotolerance [12]. *ClpB* was also induced at low temperature (25°C), and the *clpB* mutant showed decreased viability during cold stress, indicating that ClpB protected cells during cold stress in

Synechococcus [58]. *ClpC* was also cloned from *Synechococcus* [6]. It is a constitutively expressed single copy gene. The *clpC* knockout was unable to produce a viable mutant, suggesting that *clpC* was essential at any temperature [6]. A similar response was found for an *htpG* mutant [68]. In *Synechococcus*, *htpG* transcription was monocistronic. The *htpG* null mutant showed little difference in growth at 30°C and 42°C, but growth was arrested at 45°C, while wild type cells could grow at this temperature. The mutant also lost basal and acquired thermotolerance, suggesting that HtpG could protect cells during heat stress [68]. HtpG in *Synechococcus* also protected cells during cold stress [24]. There are three *dnaK* homologs (*hsp70*) in *Synechococcus* sp. strain PCC 7942. DnaK2 proteins increased after heat shock, but DnaK1 and DnaK3 were not heat inducible. DnaK1 protein actually decreased after heat shock. Mutagenesis experiments showed that DnaK2 and DnaK3 were essential for normal growth, but DnaK1 was not essential [49]. In *Synechocystis* sp. PCC 6803, *hsp16.6* was cloned and characterized. After heat stress, the *hsp16.6* null mutant showed a decrease in cell growth, oxygen evolution rates, basal thermotolerance, and acquired thermotolerance [38, 37]. In addition, ultrastructural studies showed damage to thylakoids after heat stress [37]. The *htpG* single null mutation and double mutation (*ΔhtpG::hsp16.6*) showed decreased basal and acquired thermotolerance [14]. In *Synechococcus* sp. PCC 7942, a novel heat shock gene, *orf7.5* encoding 63 amino acids was mutated [45]. The null mutant grew more slowly than wild type at 45°C, and lost basal and acquired thermotolerance. Interestingly, the *orf7.5* gene mutation also decreased *groESL* transcription, suggesting that *orf7.5* may have a regulatory role [45]. Table 2 shows the list of heat shock genes that have been mutated in cyanobacteria.

Heat shock gene overexpression in cyanobacteria results in increased thermotolerance [45]. The *clpB* gene sequence is conserved and all bacterial *clpB* genes studied so far have dual translation. There is a full-length 93-kDa protein (ClpB-93) and a truncated 79-kDa form (ClpB-79). The *clpB* from *Synechococcus* sp. PCC was expressed in *E. coli* cells. The truncated ClpB-79 form conferred the same basal thermotolerance at high temperatures in *E. coli* cells [7]. Another heat shock gene, *dnak1*, from the cyanobacterium, *Aphanothece halophytica*, was expressed in tobacco plants. Under non-stressed conditions, the transgenic tobacco had a similar growth rate compared to control tobacco; however, during salt stress, the expression of *dnaK1* increased salt tolerance [65]. Overproduction of DnaK1 or DnaK2 (but not DnaK3), from *Synechococcus* PCC 7902 resulted in defects in *E. coli* cell septation and formation of cell filaments [49]. In an *E. coli* dnaK756 mutant, dnaK2 suppressed the growth deficiency at the nonpermissive temperature, but dnaK1 and dnaK3 overexpression inhibited cell growth at the permissive temperature. The results suggest that even in the cellular compartment, different types of DnaK have specific roles [49]. Besides *hsp100* and *hsp70* homologs, the *hsp60* homolog, *groEL,* had a similar protective effect when expressed in *E. coli*. Two genes, *groEL* and *cpn60*, from *Synechocystis* PCC 6803 were expressed in the *E. coli groEL* null mutant. *Synechocystis groEL* expression restored cell growth at high temperature, mature bacteriophage T4 formation, and active Rubisco enzyme assembly, but *cpn60* overexpression only played a partial role for growth at high temperature and active Rubisco enzyme assembly. *Cpn60* did not help form mature bacteriophage T4. This suggested that GroEL and Cpn60 may have different functions in *Synechocystis* cells [32]. Several *smhsps, smhsp* from the brine shrimp, *Artemia franciscana*, *oshsp*

from rice, *Oryza sativa* cytoplasm, *tom111* from tomato, *Lycopersicon esculentum* chloroplasts, and *hsp16.6* from cyanobacterium, *Synechocystis* sp. PCC 6803, have been expressed in *E. coli* [57]. All three proteins could protect malate dehydrogenase (MDH) and *E. coli* soluble protein from thermal aggregation during heat shock. These proteins also have protective roles on *Synechococcus* PCC 7902 soluble proteins and phycocyanin. However, the smHSP did not enhance thermotolerance of PSII in isolated thylakoid membrane from S*ynechococcus* PCC 7902 [57]. The *hspA* gene from *Synechococcus vulcanus*, a small *hsp*, has a high identity with *hsp16.6* from *Synechocystis* sp. PCC 6803 [45]. *Synechococcus* sp. PCC 7942 transformed with *hspA* had higher viability when treated at 50°C in the light, but not in the dark. Expression of *hspA* in *Synechococcus* sp. PCC 7942 increased photosystem II thermotolerance and protected phycocyanin during heat stress [45].

Recent studies showed that heat shock proteins function in cells as a network [70, 71]. For newly synthesized proteins, DnaK binds to a nascent peptide with the help of DnaJ and ATPase. The complex is then delivered to the GroEL chaperone system. The nascent peptide is released from the GroEL-protein complex with the help of ATPase, and then maintains a native functional conformation. Under stress conditions, the DnaK/DnaJ/GrpE, smHSP and GroEL/ES major chaperone systems bind to active proteins to maintain proteins in a native conformation. Denatured proteins are bound to DnaK/DnaJ/GrpE to be renatured or delivered to GroEL/ES for renaturation. Denatured proteins are degraded by ClpB/C [70, 71]. In cyanobacteria, heat shock protein interaction is considered similar to other prokaryotes from *in vitro* analysis. Recombinant

Synechocystis HSP16.6 bound to denatured malate dehydrogenase (MDH), and delivered the complex to the DnaK/DnaJ/GrpE and GroEL/ES for subsequent renaturation [71].

Heat shock gene regulation

In *E. coli*, most heat shock genes are controlled by sigma factor 32 (δ^{32}), the product of the *rpoH* (*htpR*) gene [41, 56, 60]. The δ^{32} regulon consists of more than 30 heat shock genes. Another regulon δ^{E}, consists of 10 heat shock genes, while δ^{54} consists of the *psp* operon in *E. coli* [1, 2]. δ^{32} is an unstable protein and has 10-30 copies per cell at 30°C. During heat shock, δ^{32} directs the RNA polymerase core enzyme to the δ^{32} type promoter and replaces δ^{70}, which is heat-aggregated and inactivated. This binding of δ^{32} to the heat shock promoter results in the induction of heat shock genes, such as *dnaK/dnaJ*, *groESL* genes. The accumulated DnaK/DnaJ proteins associate with δ^{32} and prevent its interaction with the RNA polymerase core enzyme, then target δ^{32} for proteolytic degradation. At the same time, the DnaK chaperone complex disaggregates and reactivates δ^{70}, which binds the RNA polymerase core enzyme to replace δ^{32}. This attenuates heat shock genes, and resumes the expression of housekeeping genes [44, 56]. In *Bacillus subtilis*, four different types of heat shock regulation have been classified [21, 47, 73]. Class I genes such as the *groESL* operon have a conserved inverted repeat (IR) called CIRCE (Controlling Inverted Repeat of Chaperone Expression) element near the transcriptional start point. At low temperature, the repressor HrcA (heat regulation at CIRCE) binds to the CIRCE sequence, and controls the basal level of downstream operon transcription. When the temperature increases to a certain level, the repressor dissociates from the CIRCE element, and transcription of the downstream operon dramatically

increases. The second class of heat shock genes involves the alternative sigma factor, δ^B, which regulates more than 100 heat shock genes. Class III heat shock genes, such as *clpC*, *clpP*, and *clpE* are negatively controlled by another repressor CtsR (Class Three Stress Gene Repressor). For all other heat shock genes, the mechanism of regulation is unknown. The unknown mechanism is placed in a fourth regulation category [2, 47].

δ^{32} was not found in *Synechocystis* cells when we searched the complete genome database (http://www.kazusa.or.jp/cyanobase/). Therefore, the δ^{32} and δ^{70} switch regulon during heat shock is not present in *Synechocystis*. We have found nine sigma factors (Sig A, B, C, D, E, F, G, H, I) using "Sig" keyword to search the *Synechocystis* genome database. The mutation of three sigma factors (*sigF, G, H*) and a regulatory gene *rsbU* did not affect the expression of some heat shock genes during heat shock [25]. This suggests that these three sigma factors and *rsbU* did not control these heat shock genes directly [25]. The CIRCE element was also found in cyanobacteria. The conserved *groESL* operon was characterized in *Synechocystis* PCC 6803 [17]. The *groESL* transcriptional start point was determined by primer extension. A single transcriptional start point was found 74 bp upstream of ATG translational start codon of *groESL*. The transcriptional start point was also determined to be 73 bp upstream of the translation start codon in the *hsp60* family, *cpn60* (chaperonin 60). Since the cDNA length for primer extension was the same at normal and heat shock temperatures, no other promoters were present upstream of *groESL* and *cpn60*. The *Synechocystis groESL* operon also has a CIRCE element. The sequence is 5'-*TTAGCACTC*GTGAGGTGG*GAGTGCTAA*-3'. The inverted repeat is 9 bp (*TTAGCACT C*--------*GAGTGCTAA*) and are separated by 9 bp (GTGAGGTGG). The transcriptional

start point (G, underlined) of *groESL* is located within the CIRCE element. A similar CIRCE element for *cpn60* is 5'-*TTAGCACTC*CACTGCCAA*GAGTGCTAA*-3' (the transcriptional start point, A, is underlined) [17].

The CIRCE element is the binding site of the HrcA repressor [47, 63]. During normal conditions, the HrcA repressor binds to the inverted repeat sequence, and controls gene expression. Upon heat shock, HrcA is dissociated from the IR sequence. The dissociation of HrcA results in the induction of downstream genes [47]. We have found the *hrcA* ORFs for *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120, and *Thermoynechococcus elongatus* BP-1, from Cyanobase. They share high identity with *hrcA* from other prokaryotes. Although a detailed study has not been conducted in cyanobacteria, we propose that the same regulatory mechanism may be present in cyanobacteria due to the highly conserved region of the CIRCE element and HrcA.

Another promoter also has been proposed in cyanobacteria [45, 62]. A *smhsp*, called *hspA*, encoding a 16 kD protein, was characterized in *Synechococcus Vulcanus* [61, 62]. The mRNA was monocistronic, and primer extension located the transcriptional start point 121 bp upstream from the translational start point. There are no alternative transcriptional start points present. Sequence analysis revealed that the -35 (TTGTAT) and -10 (TATAGT) are similar to the *E. coli* δ^{70}-dependent promoter [62]. The heat shock promoter recognized by δ^{32} (controlling *smhsp ibpA/B* in *E. coli*) and CIRCE elements were not found [62]. *OrfY* preceding *hsp18* in *Streptomyces albus*, and involved in regulating *hsp18* [28], was not found upstream of *hspA* [62]. A *hspA* homolog was identified in *Thermosynechococcus elongatus* [30]. Using gel mobility shift, an AT-rich imperfect inverted-repeat sequence (ACAAgcAAA-TTTagTTGT) between the *hspA*

translational start codon and transcriptional start site was a binding target for a protein in the extract of unstressed cells. Point mutation in the inverted-repeat sequence eliminated DNA-binding activity [30]. The DNA-binding activity in the cell was lost using a protein extract from the heat shocked cells. No DNA-binding activity was observed using a protein extract from *Synechocystis* sp. PCC 6803 or *Synechococcus* sp. PCC 7942. It is proposed that the inverted repeat (ACAAgcAAA-TTTagTTGT) plays an important role in regulating *hspA* expression in *Thermosynechococcus elongatus*. A working model for *hspA* regulation has been proposed [30]. At normal growth temperature, a repressor binds to the inverted repeat sequence to repress the *hspA* expression. During heat shock, the dissociation of repressor-DNA binding resulted in the induction of *hspA* [30]. The comparison of the putative non-CIRCE promoters of heat shock genes in cyanobacteria and *smhs*p is shown in table 3. The inverted and direct repeat sequence controlling *hsp* expression in eubacteria is shown in Table 4.

In our lab, *hsp16.6* was cloned and mutated [37, 38]. Functional analysis showed *hsp16.6* protects cells during heat stress [37, 38]. In this dissertation research, Chapter 2 presents the study of the *hsp16.6* upstream region. To elucidate *htpG* function, *htpG* was mutated and the effects of mutation on thermotolerance were examined in *Synechocystis* sp. PCC 6803. The results are presented in Chapter 3. An appendix contains information not included in the two manuscripts (Chapters 2 and 3).

Table 1. Heat shock genes in cyanobacteria.

Genes	Organisms	Induction	Operon	References
clpB (*hsp100*)	*Synechococcus* sp. PCC 7942	Induced by heat shock when shift from shift from 37°C to 47.5°C, and 50°C; Induced at low temperature when shift from shift from 37°C to 20°C, and 25°C	Single copy, dual translation	[6, 58]
clpC	*Synechococcus* sp. PCC 7942	Constitutively expressed; Non inducible	Single copy, and monocistronic	[6]
htpG (*hsp90*)	*Synechocystis* sp. PCC 6803	About 1.2 fold when shift from 30°C to 42°C for one hour	Single copy, and monocistronic	
htpG (*hsp90*)	*Synechococcus* sp. PCC 7942	About 20 fold when shift from 30°C to 45°C for 15 minutes	Single copy and monocistronic	[68]
dnaK (*hsp70*)	*Synechocystis* sp. PCC 6803	Maximal level 25 fold when shift from 20°C to 42°C for 1.5 hours	Single copy, and monocistronic	[5, 15]
dnaK1 operon (*hsp70*)	*Aphanothece halophytica*	Inducible	Comprised operon of *grpE-dnaK1, dnaK1, dnaJ*, and *grpE*	[35]
Triple *dnaK* genes (*hsp70*)	*Synechococcus* sp. PCC 7942	*DnaK2* heat inducible, *dnaK1* and *dnaK3* not	Comprised *dnaK* family	[49, 50, 51]

groEL operon (*hsp60*)	*Synechocystis* sp. PCC 6803	Maximal level 110 fold when shift from 30°C to 42°C for 1.5 hours	bicistronic	[32, 40]
groESL operon (*hsp60*)	*Synechococcus vulcanus*	Inducible	operon	[67]
groESL operon (*hsp60*)	*Synechococcus* sp. PCC 7942	Maximal level 120 fold when shift from 30°C to 45°C for 20 minutes	Bicistronic operon	[75]
groESL operon (*hsp60*)	*Anabaena* sp. strain L-31	Induced when shift from 27°C to 42°C	Bicistronic operon	[59]
groEL (*hsp60*)	*Synechococcus vulcanus*	Inducible	Single copy, and monocistronic	[16]
cpn 60 (*hsp60*)	*Synechocystis* sp. PCC 6803	Maximal level 30 fold when shift from 20°C to 42°C for 1.5 hours	Single copy, and monocistronic	[5]
hsp16.6 (*smhsp*)	*Synechocystis* sp. PCC 6803	About 30 fold when shift from 27°C to 42°C for one hour	Single copy, and monocistronic	[38]
hspA (*smhsp*)	*Synechococcus vulcanus*	About 10 fold when shift from 50°C to 65°C for 15 minutes	Single copy and monocistronic	[61]
orf7.5	*Synechococcus* sp. PCC 7942	About 3-4 fold when shift from 30°C to 40°C, and 4-9 folds when shift from 30°C to 45°C for 15 minutes	Single copy and bicistronic	[46]

Table 2. Effects of heat shock gene mutation in cyanobacteria.

Genes	Organisms	Effect of gene mutation	References
clpB	*Synechococcus* sp. PCC 7942	Lost basal and acquired thermotolerance	[12]
clpC	*Synechococcus* sp. PCC 7942	Failure for mutation, suggesting *clpC* is essential in cells	[6]
htpG	*Synechococcus* sp. PCC 7942	Grows well at 30°C and 42°C; Growth defect at 45°C; Lost basal and acquired thermotolerance; Lost resistance for cold stress	[24, 68]
htpG	*Synechocystis* sp. PCC 6803	Grows well at 30°C and 37°C; Growth defect at 40°C; Lost basal and acquired thermotolerance	[14]
hsp16.6 and *htpG* double mutant	*Synechocystis* sp. PCC 6803	Lethal at mildly high temperature; Grows well at 30°C and 37°C; Growth defect at 40°C; Lost basal and acquired thermotolerance	[14]
DnaK1, 2, 3 family	*Synechococcus* sp. PCC 7942	*DnaK1* not essential, while *DnaK2, 3* essential for normal growth	[49]
hsp16.6	*Synechocystis* sp. PCC 6803	Grows well at 30°C and 37°C; Growth defect at 40°C; Lost basal and acquired thermotolerance	[37, 38]
orf7.5	*Synechococcus* sp. PCC 7942	Lost basal and acquired thermotolerance; May regulate *groESL* operon expression	[46]

Table 3. List of the putative non-CIRCE promoters of heat shock genes in cyanobacteria and *smhsps*.

Genes	Organisms	Sequence upstream of TSP*	Reference
Hsp16.6	*Synechocystis* sp. PCC 6803	CGAATCTAACCTGGAAGGGGAAATTTTAAGATAGAACCA	This work
Orf7.5	*Synechococcus* sp. PCC 7942	TTGAGAACAGCCCTCGACGAAATGA GAAGATTACGAAT	[46]
HspA	*Synechococcus Vulcanus*	TTGTATCAAGACTTGCATCCTC TATAGTGAAGGTG	[62]
HspA	*Thermosynechococcus elongatus*	+40 ACAAgcAAA-TTTagTTGT +57	[30]
Hsp18	*Leuconostoc oenos*	TTGACTTTCTGACCAATACTGAC TATTATAATAACTG	[28]
δ^{32} promoter consensus	*B subtilis*	AAATC 14, 15 bp TANTGNTTNTA	[9]
E. coli δ^{32} promoter consensus	*E. coli*	TNtCNCcCTTGAA 13-15 bp CCCCATtTa	[9]
E. coli δ^{70} promoter consensus	*E. coli*	TTGACA 16-18 bp TATAAT	[9]
		-35 -10 TSP*	

*TSP, transcriptional start point

Table 4. The inverted and direct repeat sequences for *hsp* regulation in eubacteria.

DNA element	Sequence	Repressor	Regulated genes or operons	Organisms	Reference
CIRCE	TTAGCACTC-9 bp-GAGTGCTAA	HrcA	*groESL and dnaK operons*	Many eubacteria	[21, 30, 47]
HAIR	CTTGAGT-7 bp-ACTCAAG	HspR	*dnaK operon, clpB*	*Streptomyces albus*	[21, 30, 47]
-	TGTCATC-5 bp-GATGACA	RheA	*Hsp18*	*Streptomyces albus*	[30]
	ACAAgcAAA-TTTagTTGT	Unknown	*hspA*	*Thermosynecho coccus elongatus*	[30]
Direct repeat	A/GGTCAAA-NAN-A/GGTCAAA	CtsR	*clpC, clpP*	*Bacillus subtilis,* and *Listeria monocytogenes*	[10, 30, 34, 43]

Revised from Narberhaus (1999), Kojima and Nakamoto (2002).

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Expression of the Heat Shock Gene *Hsp16.6* and Promoter Analysis in the Cyanobacterium, *Synechocystis* sp. PCC 6803

Abstract

Hsp16.6 and its upstream region from the cyanobacterium, *Synechocystis* sp. PCC 6803, has been analyzed. The *hsp16.6* transcriptional start point was positioned 44 base pairs (bp) upstream of ATG translation start codon. A reporter vector was constructed by ligating the 265 bp upstream fragment onto the upstream region of the *lacZ* coding sequence. β-galactosidase analysis indicated the 265 bp region did not induce *lacZ* gene expression in *E. coli*; although *lacZ* expression was induced when the *Synechocystis groESL* promoter was used. In *Synechocystis* cells, *lacZ* was expressed when the 265 bp fragment was used as a promoter. Cold stress and ethanol did not induce *lacZ* expression, while heat shock, salt stress, sorbitol, hydrogen peroxide and high light induced *lacZ*. Series deletions from the 265 bp region demonstrated that the induction of β-galactosidase activity was lost when a region around -35 was deleted.

Keywords: Heat shock, cyanobacteria, *hsp16.6*, regulation, reporter, promoter.

Introduction

The heat shock response is a protective adaptation found in all living organisms. When organisms are exposed to elevated temperatures, heat shock proteins (HSPs) are expressed transiently to protect them from heat damage. During heat stress, heat shock proteins increase dramatically, but at normal growth temperatures, these proteins are present in low concentrations [16, 24].

HSPs are divided into five conserved classes according to size: HSP100, HSP90, HSP70, HSP60 and small (smHSP) or low molecular weight (LMW) HSPs (below 30 kD) [16, 24]. In cyanobacteria, smHSPs have been studied recently. In *Synechocystis* cells, the null mutant of *hsp16.6* decreased basal and acquired thermotolerance [10, 11]. *In vitro* functional analysis showed that *Synechocystis* HSP16.6 bound to denatured malate dehydrogenase and transferred the complex to the DnaK/DnaJ/GrpE and GroEL/ES chaperones for subsequent renaturing [23]. *In vivo* and *in vitro* studies also showed that HSP16.6 oligomerization is essential for chaperone activity [3]. The expression of the *hsp16.6* homologue, *hspA*, from the thermal cyanobacterium, *Synechococcus vulcanus*, in *Synechococcus* sp. PCC 7942 cells increased thermal resistance of photosystem II (PS II) and protected phycocyanin [14, 19, 20].

In prokaryotes, heat shock gene regulation has been well-studied in *E. coli* and *Bacillus subtilis* [1, 6]. In *E. coli*, most heat shock genes are controlled by sigma factor 32 (δ^{32}), the product of the *rpoH* (*htpR*) gene [1]. The δ^{32} regulon consists of more than 30 heat shock genes. Another regulon, δ^{E}, consists of 10 heat shock genes and δ^{54} consists of the *psp* operon in *E. coli* [1]. In *Bacillus subtilis*, four different types of heat shock regulation have been classified [1, 6, 13, 22]. Class I genes such as *groESL* operon have a

conserved inverted repeat (IR) called CIRCE (Controlling Inverted Repeat of Chaperone Expression) element near the transcriptional start point. At the growth temperature, the repressor HrcA (heat regulation at CIRCE) binds to the CIRCE sequence, and controls the basal level of downstream operon transcription [1, 6, 13, 22]. The second class of heat shock genes involves the alternative sigma factor, δ^B, which regulates more than 100 heat shock genes. Class III heat shock genes, such as *clpC*, *clpP*, and *clpE*, are negatively controlled by another repressor CtsR, (Class Three Stress Gene Repressor). For other heat shock genes, the mechanisms of regulation are unknown, and thus, constitutes a fourth category of regulation [1, 6, 13, 22].

Although the function of smHSP has been studied in cyanobacteria, *smhsp* gene regulation is not understood. Since *Synechocystis* is a photosynthetic organism, light and the photosynthetic inhibitor, DCMU, can influence the expression of heat shock genes [4]. This suggests that heat shock gene regulation is different from other prokaryotes. In *Synechocystis* cells, δ^{32} was not found when the whole genomic database was searched in Cyanobase (http://www.kazusa.or.jp/ cyanobase/). This suggests that although δ^{32} is a main heat shock regulatory factor in *E. coli*, this type of regulation is not present in *Synechocystis* PCC 6803. From the *Synechocystis* genome database, nine sigma factors (Sig A, B, C, D, E, F, G, H, I) have been found using "Sig" keyword to search the whole genome using Cyanobase. The mutation of three sigma factors (*sigH, G, F*) and a regulatory gene, *rsbU*, did not affect *hsp16.6* gene expression during heat shock or high light [7]. Only a mutant in *sigF* decreased *hsp16.6* gene expression slightly after salt stress [7]. Similar work showed that the *sigC*, *sigD*, *sigE*, and *sigF* mutations did not affect the induction of *hsp16.6* by heat; however, *sigB* mutation resulted in a decrease in

the heat induction level of *hsp16.6* [8]. In *Synechocystis* sp. PCC 6803, the CIRCE element is present upstream of the *groESL* operon [12], but no CIRCE element was found upstream of the *hsp16.6* coding sequence. How *hsp16.6* is regulated is unknown. Recently, an AT-rich imperfect inverted-repeat (ACAAgcAAA-TTTagTTGT) upstream of *hspA*, the *hsp16.6* homologue, was demonstrated to have binding activity with total protein isolated from the cyanobacterium *Synechococcus vulcanus* [9]. However, this sequence is absent in the upstream region of *hsp16.6*.

In this study, we mapped the transcriptional start point of *hsp16.6*. A 265 bp region upstream from the *hsp16.6* gene was fused with a *lacZ* reporter. The dissection of the 265 bp fragment suggested that a sequence around -35 is important for *hsp16.6* heat induction.

Material and methods

Organisms

Synechocystis sp. PCC 6803 cells were grown in BG-11 medium at 30°C under constant fluorescent illumination (40 μE.m^{-2}.sec^{-1}) [25]. For the *Synechocystis* mutant, the antibiotic, chloramphenicol, was added to the BG-11 medium to a final concentration of 5μg/ml in liquid medium and plates. For *E. coli*, JM109, JM110, and CM404 strains, cells were grown in LB supplemented with 100 μg/ml ampicilin, 50 μg/ml kanamycin, and 34 μg/ml chloramphenicol, as required [21].

Total RNA isolation and RT-PCR

Synechocystis cells were heat shocked at 42°C for one hour in light in a shaking water bath to induce heat shock genes. Cells were lysed and total RNA was isolated from cells. The isolated total RNA was treated by RNase-free DNase at 37°C for 1 hour in the presence of an RNase inhibitor [11]. The treated RNA was then purified and total RNA was used for Northern blotting, RT-PCR, and primer extension. RT-PCR was performed according to the protocol provided by Promega (Madison, WI, USA). One μg of total RNA was used as a template for RT-PCR, and primers for the *hsp16.6* coding sequence amplification were used.

Primer extension

A primer (5'-CCGTAGGGACAAAAACTTCTT-3') 91-70 bp downstream of the ATG start codon of *hsp16.6* gene was labeled with γ-P_{32} dATP using T4 polynucleotide kinase [21]. Primer extension was performed at room temperature, and the reaction was loaded onto a sequencing gel. Manual sequencing was conducted according to the

protocol provided by Amershan (Piscataway, NJ, USA). The pGEM-T vector was ligated to the *hsp16.6* gene coding sequence, and used for generating sequencing ladders.

Reporter construction

Two primers (forward, 5'-CGGAATTCGCAGTGAAGGATCTAGTCGG-3', with an *Eco*RI site underlined; reverse primer, 5'-CATG*CC*ATGGACTAG TCATCCTCCAGATGATA-3', with a *Nco*I site underlined) were designed to amplify 170 bp of the *groESL* promoter from *Synechocystis*. Two primers (forward, 5'-CGGAATTCTTTTTCACAACCTAAGAC TTC-3', with an *Eco*RI site underlined; reverse primer, 5'-CATG*CC*ATGGTGTTAACTCCTGATGT-3', with a *Nco*I site underlined) were designed to amplify 265 bp (designated p265 fragment) of the *hsp16.6* upstream region from *Synechocystis* genomic DNA.

The *hsp16.6* sequence around the ATG translational start codon (5'-ACATCAG GAGTTAACATTATGTCTCT-3') was changed to create an *Nco*I restriction site using the reverse primer (5'-TGTAGTCCTCAATTGTGGTACCGTAC-3') to ligate the p265 fragment into pL526D plasmid This change did not alter the *lacZ* coding sequence, and still retained the Shine-Dalgarno sequence (AGGAGT). The 265 bp PCR fragment and plasmid, pL526D, were digested with *Eco*RI and *Nco*I. The p265 fragment was then ligated upstream of the *lacZ* coding sequence, and the reporter construct (designated pPSM265) was transformed into *E. coli* JM110 cells (*dam* methylase mutation). β-galactosidase activity was analyzed in *E. coli* cells harboring the reporter construct according to the protocol provided by Promega (Madison, WI, USA).

Shuttle vector construction

Two primers (forward, 5'-TACTGCAGTTCGAAGCATCTAATCGCTTGA GTTA-3', with *Pst*I and *Csp*45I restriction sites underlined; reverse, 5'-ACGGTACCAGGCCCTATCGATCAATTCGGGCACGAACCCAG-3', with *Kpn*I, *Eco*O109I, and *Cla*I restriction sites underlined) were used to amplify the 1 kb chloramphenicol resistance cassette (Cm^r) from pPZP111 [5]. PCR was performed using Vent DNA polymerase to produce a blunt-end and high fidelity PCR product (New England Biolab, Beverly, MA, USA). Plasmid pKT210 (RSF1010-derived plasmid) [2] was digested with *Xmn*I and *Pst*I to delete the 3.5 kb fragment. One end of the Cm^r cassette was digested with *Pst*I, and ligated into the 5783 bp *Xmn*I-*Pst*I linearized pKT210 to construct a shuttle vector (designated pRSF). The plasmid, pRSF, was digested with *Csp*45I to linearize the molecule, and the two ends were partially filled to produce a 5'-C overhang by adding ATG, TTG, and CTG without GTG in the presence of DNA Polymerase I Large (Klenow) Fragment. The plasmid, pPSM265, was digested with *Eco*O109I and the ends were partially filled to produce a 5'-G overhang by adding ATG, TTG, and GTG without CTG. The 5.4 kb DNA fragment was ligated into the *Csp*45I linearized plasmid, pRSF. The reporter shuttle vector (designated pRES) was transformed into *E. coli* JM109 cells, and transformants were selected on LB plates with chloramphenicol and X-gal. Light blue colonies were picked for construct confirmation.

P265 deletions

Forward primers p2, p3, p4, p5, and p6 (see Table 2, and Fig. 10), and reverse primer (5'-TGTAGTCCTCAATTGTGGTACCGTAC-3') were used to generate deletions of p265 to create *lacZ* fusion constructs controlled by deletions in p265. The

PCR products were digested with *Eco*RI and *Nco*I, then ligated to pL526D linearized by *Eco*RI and *Nco*I. The deletion constructs were then ligated to the pRSF plasmid as described previously. The resulting constructs were then transformed into *Synechocystis* cells.

Triparental conjugation

Triparental conjugation was conducted according to a modification of Williams [25]. *E. coli* JM109 cells carrying the shuttle vector and CM404 cells (HB101 carrying helper plasmid pRK2031) were cultured overnight. One ml each of JM109 and CM404 cells was centrifuged at 12,000g for one minute. The pellets were washed with LB once, and mixed together in 50 μl of LB. One ml of mid-exponential culture of *Synechocystis* cells was centrifuged at 12,000g for one minute, and resuspended in 50 μl of fresh BG-11 medium. The three cell suspensions were mixed together in an Eppendorf tube, and wrapped with Parafilm. The mixture was placed in an incubator at 30°C under light for 20 hours to develop chloramphenicol resistance. The suspension was then spread onto BG-11 agarose plates with 5μg/ml chloramphenicol. After five weeks, green colonies were selected to grow in BG-11 liquid medium supplied with 5μg/ml chloramphenicol.

Total DNA was isolated from *Synechocystis* cells using Puregene DNA purification (Gentra systems, Minneapolis, MN, USA) to confirm transformation. Purified DNA was used as a template for PCR (forward primer 5'-CGGAATTCTTTT TCACAACCTAAGACTTC-3' to amplify the p265 fragment; reverse primer 5'-CACCACAGATGAAACGCCGAG-3', located 439-451 bp downstream of the *lacZ* ATG translational start codon).

Stress treatment on cells

Cells were grown at 30ºC, and heat shocked at 42ºC to induce the fusion reporter. For cold treatment, cells were placed in a 15ºC water bath for 1 hour. For high light treatment, cells were exposed to a light intensity of 300 $\mu E.m^{-2}.sec^{-1}$ for 1 hour. Ethanol (5%), NaCl (0.684 M), sorbitol (0.5 M) or H_2O_2 (200 μM) was directly added into cell cultures for 1 hour [7]. After treatment, cells were washed with fresh BG-11 medium one time. The cells were then lysed for β-galactosidase analysis.

β-galactosidase activity

A β-galactosidase analysis kit (Promega, Madison, WI, USA) was used to determine promoter activity. *E. coli* cell lysis was conducted according to technical communication from Promega. *E. coli* cells were grown at 30 °C, and heat shocked at 42 °C for 1 hour. One ml of cells were centrifuged at 12,000×g for one minute, and the pellet was washed with one ml of PBS buffer. Cells were centrifuged at 12,000g for 1 minute, resuspended in lysis buffer (4 mg/ml lysosome in 50 mM glucose, 10 mM EDTA, 25 mM Tris, pH=8), and incubated at room temperature for 1 hour. Cells were centrifuged at 12,000g for one minute and resuspended in 200 μl of lysis buffer. For β-galactosidase analysis, *Synechocystis* cells were heat shocked at 42 °C for 1 hour and centrifuged at 12,000g for two minute. Pellets were washed once with PBS buffer and resuspended in lysis buffer. Cells were sonicated three times (30 seconds each time at output of 100 watts). The lysate was centrifuged at 12,000g for 10 minutes, and the supernatant was used for the measurement of β-galactosidase activity by spectrophotometery at OD_{420nm}.

Results

The *hsp16.6* expression under stress conditions

Prior to high temperature treatment, transcription of *hsp16.6* was undetectable during normal growth conditions using Northern hybridization [11]. To quantify and compare the *hsp16.6* transcriptional level during optimal growth and heat shock, RT-PCR was conducted. Results showed that *hsp16.6* transcription during optimal growth was detectable but very low (Fig. 1A, lane 2), and increased after heat shock (Fig. 1A, lane 3).

Western blotting showed that cold stress at 15ºC for 1 hour did not induce HSP16.6 expression (Fig. 1B). We also tried treatments at 15ºC and 20ºC for three hours, but no HSP16.6 induction was observed (data not shown). Besides heat shock, salt stress and H_2O_2 induced HSP16.6 expression (Fig. 1B).

The *hsp16.6* transcriptional start point

Primer extension was performed to determine the *hsp16.6* transcriptional start point. A primer 91-70 bp downstream of the *hsp16.6* ATG translational start codon was for primer extension. The cDNA from heat shocked cells was detected on the sequencing gel (Fig. 2A). The cDNA from primer extension was 134 base pairs, and thus, the transcriptional start point was positioned 44 base pairs upstream of the ATG translation start codon. The transcriptional start point was “A” (Fig. 2C).

From Northern blot hybridization, the *hsp16.6* mRNA was about 600 base pairs [11]. According to the sequence from the Cyanobase database, the upstream (right) gene is a hypothetical gene for ParA family protein. The downstream (left) gene is a c-type cytochrome synthesis protein. Considering the size of the *hsp16.6* coding region and the adjacent open reading frames, it is unlikely that *hsp16.6* is part of an operon. The

sequence between the stop codon (TAG) of the c-type cytochrome synthesis gene and the start codon (ATG) of *hsp16.6* is 265 base pairs.

Inverted repeats including CIRCE element play a role in heat shock regulation [15, 18]. To search for inverted repeats in the p265 fragment, the STEMLOOP function from GCG (San Diego, CA, USA) was used to identify stem-loops in the p265 DNA fragment. According to the relationship between CIRCE and promoter position, we hypothesized that stem-loops around -35 and -10 might be important for heat shock regulation. Six potential stem-loops around -35 and -10 are shown (Fig. 2B & C).

The effect of *hsp16.6* and *groESL* promoters on *lacZ* expression

To test whether p265 from *Synechocystis* was responsible for heat induction, we ligated p265 upstream of the *lacZ* coding sequence. β-galactosidase activity was measured in *E. coli* cells with the reporter construct. In heat shocked JM-pRgroESL-CIRCE cells (JM109 cells harboring plasmid pRgroESL-CIRCE), β-galactosidase induction was detected (Fig. 4A). This suggested that the *groESL* promoter region including the CIRCE element from *Synechocystis* was inducible in *E coli* cells. It also indicated that the reporter construct worked properly in *E. coli* despite several base pair changes upstream of the ATG start codon of the *lacZ* coding sequence. However, β-galactosidase activity was low and only slightly induced in heat-shocked JM-pPSM265 cells (JM109 cells harboring plasmid pRSM265) (Fig. 4B).

The construction of shuttle reporter vector

Since the reporter vector, pPSM265, did not function in *E. coli*, this reporter construct was transformed back into *Synechocystis* to test whether p265 regulated *lacZ* expression during heat shock. The vector (pRES) construction was based on plasmid

pL526D and pKT210 (Fig. 3). PCR was conducted to confirm transformation of the shuttle vector. The specific PCR primer combination only amplified the DNA fragment within the shuttle reporter construct, and did not amplify any sequence from the *Synechocystis* genome. A 0.7 kb PCR product was amplified from chloramphenicol-resistant *Synechocystis* cells, but not from wild type cells (data not shown). This indicated that triparental conjugation was successful.

β-galactosidase activity

To test whether p265 fragment induced *lacZ* during heat shock, we heat shocked *Synechocystis* cells containing the reporter construct to measure β-galactosidase activity. The activity of β-galactosidase without heat shock was low. β-galactosidase activity was induced after 5 minutes of heat shock, and activity increased with longer exposure (Fig. 4C). The fusion reporter also responded to other stresses such as heat, salt, and sorbitol. High light and H_2O_2 also induced β-galactosidase activity, but not as strongly. Cold stress and ethanol had no effect (Fig. 4D).

The deletion of -35 resulted in the loss of *lacZ* induction

To determine the basal promoter for *hsp16.6* heat induction, we constructed a series of deletions of p265 (Fig. 5E). Results showed that the deletion of the region from -265 bp to -95 bp (p2 and p3) did not affect the induction of *lacZ* (Fig. 5F). However, a several base pair deletion in the -35 region resulted in a large decrease in β-galactosidase activity (p4). A deletion of upstream of -22 (p5) resulted in the total loss of heat induction of β-galactosidase activity.

Discussion

Northern blot analysis showed that *hsp16.6* mRNA is about 600 base pairs, and *hsp16.6* transcription is monocistronic [11]. However, the regulation of *hsp16.6* was unknown. In this study, RT-PCR showed that during normal growth conditions, *hsp16.6* transcription was detectible, but maintained a low basal level. Primer extension showed that the transcriptional start point of *hsp16.6* was 44 bp upstream of ATG translation start codon. Sequence analysis showed that there was no *E. coli* δ^{32} -35 and -10 consensus heat shock sequence upstream of the *hsp16.6* gene. We used a *lacZ* gene as a reporter gene to analyze the 265 bp upstream fragment. Originally, we planned to do nested deletion in *E. coli* to determine the basal *hsp16.6* regulatory elements. However, although *lacZ* was induced by the *groESL* promoter from *Synechocystis*, p265 was not effective. This suggests that p265 from *Synechocystis* was not recognized in *E. coli*. *Synechocystis* was transformed with the shuttle reporter construct to study p265 regulation. Reporter analysis showed that β-galactosidase activity was induced. β-galactosidase activity was also increased after exposure to other types of stress. This suggests that p265 may be responsible for heat shock and other stress induction.

In *Synechocystis*, the *groESL* regulatory element includes an inverted repeat called CIRCE [12]. In eubacteria, the CIRCE regulation is universal, and highly conserved [6, 15]. Under optimal growth conditions, the repressor HrcA binds to CIRCE, and maintains *groESL* expression at a basal level. During heat shock, the repressor is released from the CIRCE element, and *groESL* transcription increases dramatically [6, 15]. The occurrence of CIRCE in cyanobacteria, Gram-positive organisms, and the α-subgroup of proteobacteria suggests that negative regulation had an early evolution. This

model of regulation may be a simple way for genes to be regulated [6, 15]. However, there is no CIRCE consensus element present upstream of the *hsp16.6* gene coding sequence. Instead, we identified inverted repeats around -35 and -10 in the 265 bp fragment, with no similarity to CIRCE. Gel mobility shift assays using total proteins isolated from cells during optimal growth and heat shock were performed to explore whether p265 was bound by some unknown factors during heat stress; however, we did not find binding to p265. Our results are in agreement with those of Hitoshi Nakamoto (personal communication).

In *Synechocystis* cells, with the exception of the *groESL* operon, regulatory mechanisms have not been characterized. In *E. coli,* the unstable factor, δ^{32}, controls most heat shock genes [15, 17]. During heat shock, δ^{32} directs the RNA polymerase core enzyme to replace δ^{70} which is heat-aggregated and inactivated. The binding of δ^{32} to the heat shock promoter results in induction of heat shock genes, including *dnaK/dnaJ*. The accumulated DnaK/DnaJ proteins associate with δ^{32} to prevent its interaction with the RNA polymerase core enzyme, and target δ^{32} for proteolytic degradation. At the same time, the DnaK chaperone complex disaggregates and reactivates δ^{70}, which binds the RNA polymerase core enzyme to replace δ^{32}, resulting in the attenuation of heat shock genes [15, 17]. Since δ^{32} was not present in *Synechocystis*, the δ^{32} and δ^{70} switch regulon is not present in the *hsp16.6* promoter. From the *Synechocystis* genome database, nine sigma factors (Sig A, B, C, D, E, F, G, H, I) have been found using the "Sig" keyword to search the whole genome. The mutation of three sigma factors (*sigF, G, H*) and a regulatory gene, *rsbU*, did not affect *hsp16.6* expression during heat shock [7]. This suggested that *sigF, G, H* and *rsbU* did not control *hsp16.6* expression directly [7].

Further studies showed that heat shock regulatory factors such as δ^{32}, δ^{C}, δ^{D}, δ^{E}, δ^{F}, δ^{G}, δ^{H}, or HrcA did not regulate *hsp16.6* during heat shock and non-heat shock conditions [7, 8]. Some inverted repeats such as CIRCE have negative a regulatory function. The deletion of CIRCE resulted in the increase of gene expression during normal growth conditions [6, 15]. In this study, the partial or total deletion of inverted repeats around the -35 region did not increase the expression of *lacZ*, suggesting that inverted repeats may not be responsible for *hsp16.6* gene regulation. A δ^{B} mutation resulted in the partial loss of *hsp16.6* heat induction. However, *hsp16.6* expression was still detected, suggesting that there are additional mechanisms regulating *hsp16.6* [8]. In this study, we determined that the p265 fragment was partially responsible for *hsp16.6* induction, and that the –35 region may be essential for *hsp16.6* expression. The mechanisms regulating *hsp16.6* are under investigation.

Acknowledgements

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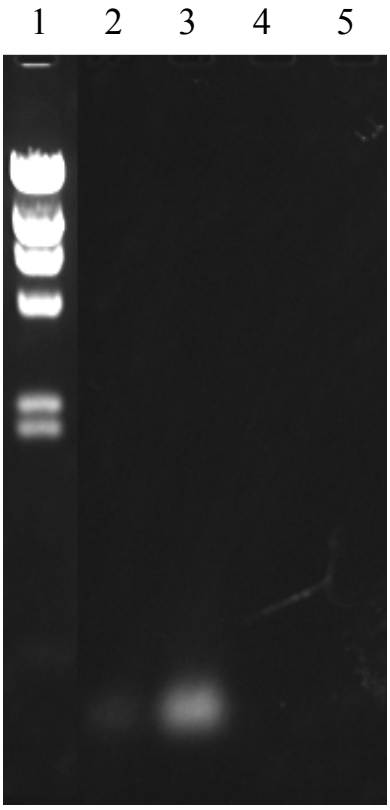
A



B

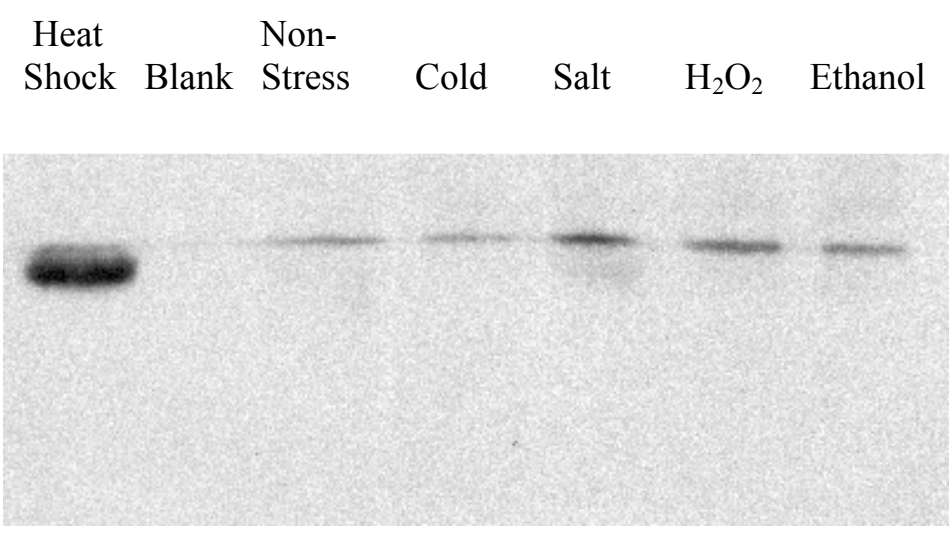
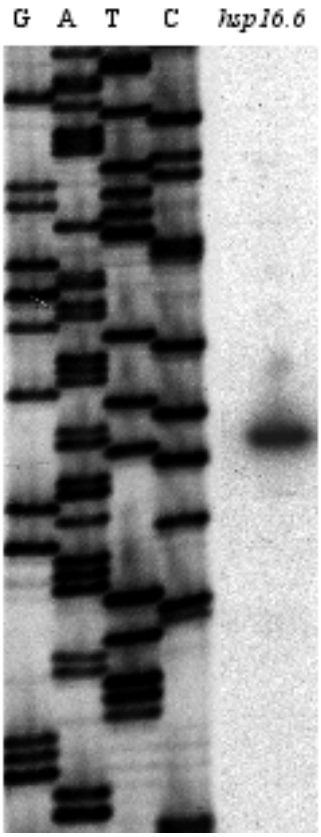


Fig. 1. The *hsp16.6* expression under stress conditions. (A) RT-PCR of *hsp16.6*. Lane 2, control at 30°C, lane 3, heat shocked at 42°C for one hour, lane 4, control at 30°C without reverse transcriptase, lane 5, heat shock at 42°C without reverse transcriptase. Lambda DNA cut with *Hin*d III as DNA marker (lane 1). (B) Western blotting, HSP16.6 expression under different stress conditions.

A



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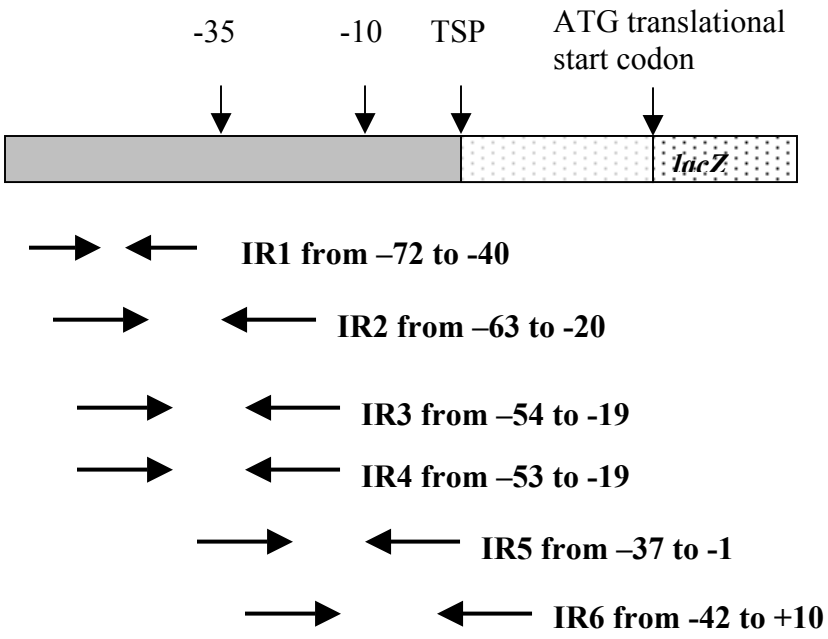


C

Sequence upstream of *hsp16.6* gene and an imperfect invert repeat (IR5) from –37 to -1

AAAACCGAATCTAACCTGGAAGGGGAAATTTTAAGATAGAACCA

-35 **-10** **TSP +1**

TTCAAGGGTAATCAATTCCTTCCACACATCAGGAGTTAACATTA***TG***

SD sequence

```
-37 GAATCTAACCTGGAA  GGG
    |  |||| | |||||     G
-1  CCAAGATAGAATTTT  AAA
```

Fig. 2. Mapping the transcriptional start point of *hsp16.6* using primer extension and sequence of p265. (A). Primer extension. The sequencing lanes from left to right are G, A, T, C. The cDNA was synthesized from total RNA isolated from heat-shocked cells at 42°C for one hour. (B). Six inverted repeats in p265. (C). Sequence upstream of *hsp16.6* gene and an imperfect invert repeat (IR6). (Figure not drawn to scale).

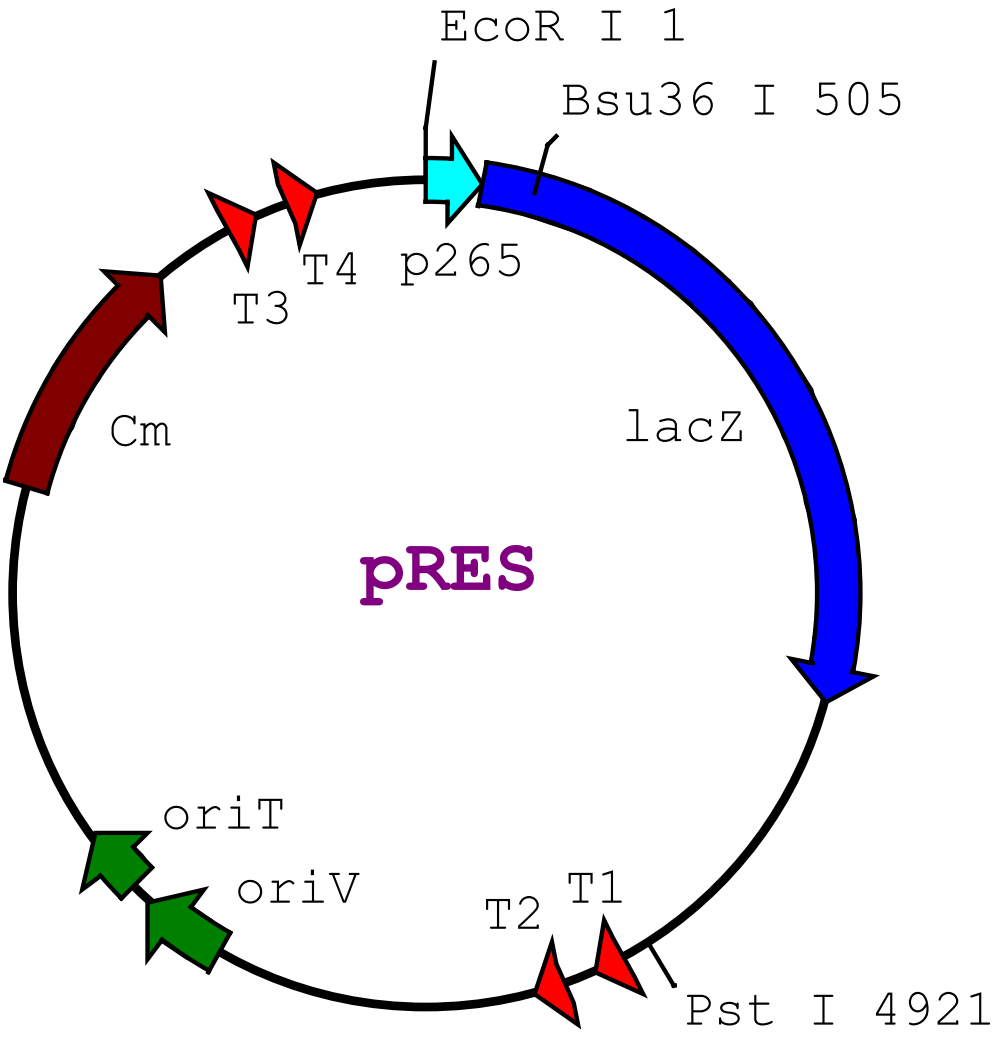
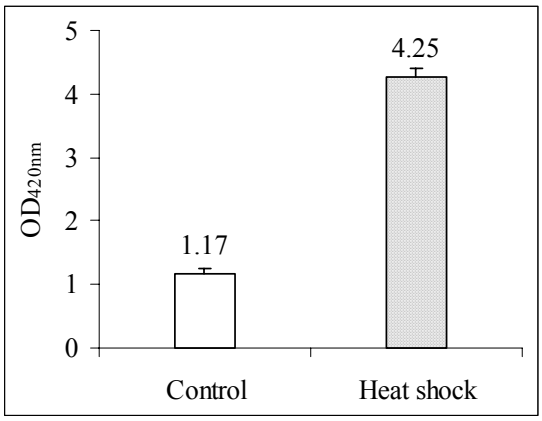

EcoR I 1
Bsu36 I 505
T4
p265
T3
Cm
lacZ
pRES
oriT
oriV
T2
T1
Pst I 4921

Fig. 3. Diagram of the shuttle vector construction (see details in material and methods).

A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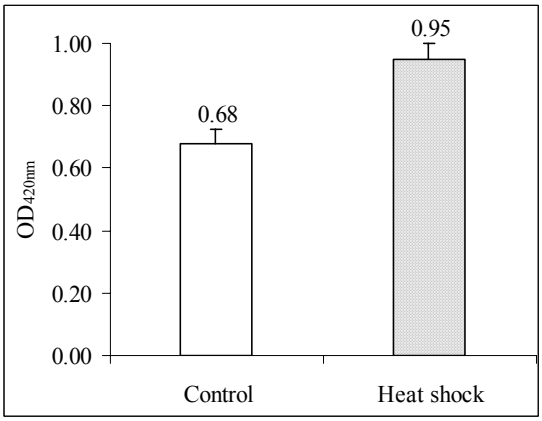
4
3
2
1
0
OD420nm
4.25
1.17
Control
Heat shock

B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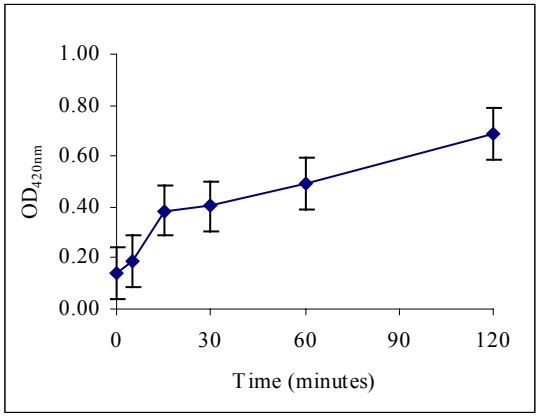
0.40
0.20
0.00
OD420nm
0.95
0.68
Control
Heat shock

C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0.60
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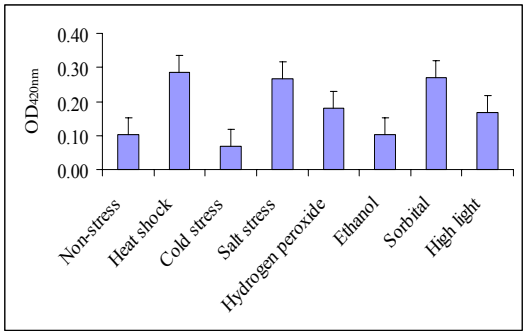
0.20
0.00
OD420nm
0
30
60
90
120
Time (minutes)

D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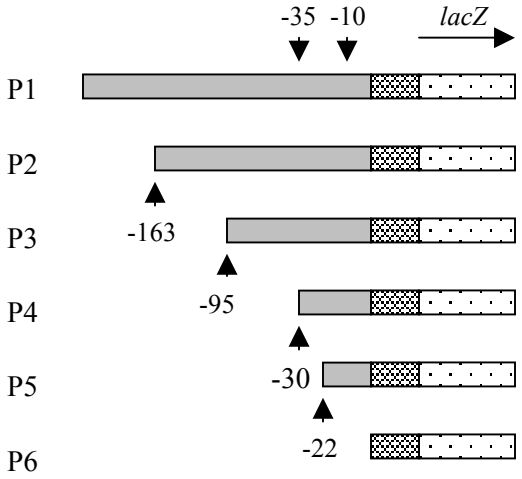
0.30
0.20
0.10
0.00
OD420nm
Non-stress
Heat shock
Cold stress
Salt stress
Hydrogen peroxide
Ethanol
Sorbital
High light

E


-35
-10
lacZ
P1
P2
P3
P4
P5
P6
-163
-95
-30
-22

F

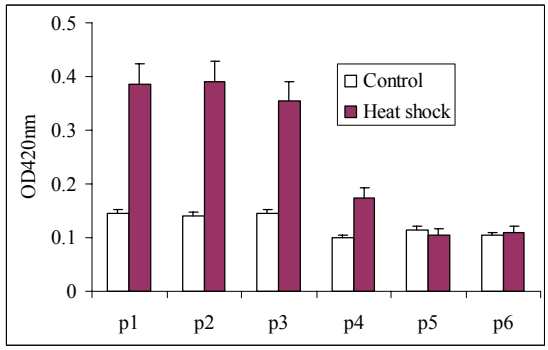

0.5
0.4
0.3
0.2
0.1
0
OD420nm
Control
Heat shock
p1
p2
p3
p4
p5
p6

Fig. 4. *LacZ* expression in cells. cells were treated, and the expression of *lacZ* was measured by β-galactosidase activity at absorbance 420 nm. (A). Control by *groESL* promoter in *E. coli* cells. (B). Control by p265 region in *E. coli* cells. (C). *Synechocystis* cells were heat shocked at 42°C. (D). *Synechocystis* cells were exposed to different stresses. (E). A series of deletion were generated by PCR using different primers (p2-p6). These were ligated upstream of the *lacZ* coding sequence. (F). β-galactosidase activity in *Synechocystis* cells transformed with the different constructs.

Table 1. Strains and plasmids used in this study

Strain	Relevant characteristics	Source or reference
JM109	For general cloning, F' traD36 proA+ proB+ lacIq delta (lacZ) M15 delta (lac-proAB) supE44 hsdR17 recA1 gyrA96 thi-1 endA1 relA1 e14- lambda-	Promega (Madison, WI, USA)
JM110	For cloning, F'[traD36 proA+ proB+ lacIq delta(lacZ)M15] dam dcm supE44 hsdR17 thi leu thr rpsL lacY galK galT ara tonA tsx delta(lac-proAB) lambda-	New England Biolabs (Beverly, MA, USA)
CM404	HB101 cells carrying conjugate plasmid pRK2013, used for the mobilization of shuttle vector, Kan^r	Lab stock
JM-PGEMT-hsp16.6	JM109 cells hosting plasmid PGEMT-hsp16.6	Lab stock
JM-pRSF	JM109 cells hosting plasmid pRSF7kb	This work
JM-pPSM265	JM109 cells hosting plasmid pPSM265	This work
JM-pRgroESL-CIRCE	JM109 cells hosting plasmid pRgroESL-CIRCE	This work
JM-pRES	JM109 cells hosting plasmid pRES	This work
JM-pRES-pUC19	JM109 cells hosting plasmid pRES-pUC19	This work
Plasmid		
PKT210	RSF1010 derivative, used to construct shuttle vector, Cm^r	[2]
pRSF	The *Pst*I-*Xmn*I fragment in pKT210 was replaced by Cm^r resistance cassette, Cm^r	This work
pGEM-T	For TA cloning, Amp^r	Promega (Madison, WI, USA)
pPSM265	pL526D derivative, p265 fragment was placed upstream of the *lacZ* coding sequence, Amp^r	This work
pRgroESL-CIRCE	pL526D derivative, *groESL* promoter was placed upstream of the *lacZ* coding sequence, Amp^r	This work
pRES	Reporter and Expression Shuttle vector, Cm^r	This work
pRES-pUC19	Hybrid of pRSE and pUC19	This work

Table 2. Primers used for deletion of p265 regions

Primer name	Primer location	Primer (*Eco*RI restriction site underlined)
Prm1	From –265 to -246	5'-CGGAATTCGCAGTGAAGGATCTAGTCGG-3'
Prm2	From –163 to –144	5'-CGGAATTCTTTTACCATTTTGTTTTGCC-3'
Prm3	From –95 to –76	5'-CGGAATTC AAAAATTGCGGCTAGAAATG-3'
Prm4	From –30 to –11	5'-CGGAATTC ACCTGGAAGGGGAAATTTTA-3'
Prm5	From –22 to –3	5'-CGGAATTC GGGGAAATTTTAAGATAGAA-3'
Prm6	From +1 to +20	5'-CGGAATTC ATTCAAGGGTAATCAATTCC-3'
PFseq33	33 bp upstream *Eco*RI site, for sequence	5'-GCCCGCCATAAACTGCCAGG-3'
Prm70	70 bp downstream of ATG translation start codon of *lacZ*	5'-CAAGGCGATTAAGTTGGGTA-3'

The Heat Shock Gene, *htpG,* and Thermotolerance in the Cyanobacterium, *Synechocystis* sp. PCC 6803

Abstract

The *htpG* null mutant was obtained by inserting a chloramphenicol resistance cassette (*Cm*^{r}) in the *htpG* coding sequence. The *htpG* null mutant (Δ*htpG*), *Δhsp16.6,* and the double mutant, *ΔhtpG::hsp16.6* cells showed little growth disadvantage at 30°C and 37°C, but not at 40°C. This suggests that HtpG and HSP16.6 proteins do not have an essential role during normal growth at normal and mildly elevated temperatures. Cell growth, cell survival rate, and oxygen electrode measurements demonstrated that *ΔhtpG, Δhsp16.6,* and *ΔhtpG::hsp16.6* cells were sensitive to heat stress. Decreased basal and acquired thermotolerance was observed when mutants were heat shocked, with *ΔhtpG::hsp16.6* being the most sensitive. A comparison of mutants showed that *Δhsp16.6* was more sensitive to heat shock than *ΔhtpG*.

Keywords: Heat shock, thermotolerance, cyanobacterium, *Synechocystis*, *htpG*, *hsp16.6*

Introduction

All organisms studied thus far respond to heat shock by the induction of heat shock proteins (HSPs). HSPs have been shown to play important roles in the protection of organisms under heat stress [13, 14]. HSPs are divided into five conserved classes according to protein size: HSP100, HSP90, HSP70, HSP60, and low molecular weight or small HSPs (smHSP, below 30 kD) [13, 14]. In prokaryotes, several heat shock proteins have been well characterized: the HSP60 homolog, GroEL (composed of GroEL-GroES chaperone complexed with HSP10 (GroES)) and the HSP70 homolog, DnaK (composed of DnaK-DnaJ-GrpE chaperone complex) [14, 20]. GroEL-GroES and DnaK-DnaJ-GrpE chaperone complexes play significant protective roles during heat stress [2, 20]. The other three classes of chaperones, ClpB (HSP100 homolog), HtpG (HSP90 homolog) and smHSP are minor chaperones [19, 20].

One of the minor chaperones, the *smhsp* gene *hsp16.6*, was characterized in *Synechocystis* PCC 6803 in our lab [10]. Physiological analysis showed that the *hsp16.6* null mutant (*Δhsp16.6*) had reduced basal and acquired thermotolerance [10], while cellular damage from heat stress was detected using transmission electron microscopy [9]. The integration of HSP16.6 into membranes could stabilize heat-stressed thylakoid membranes [5]. *In vitro* analysis showed that recombinant HSP16.6, bound to denatured malate dehydrogenase, delivered this protein-protein complex to major chaperones, GroEL-GroES and DnaK-DnaJ-GrpE, for subsequent renaturing [21].

In eukaryotes, HSP90 has molecular chaperone activity, and plays a critical role in eukaryotic signal transduction [12, 16]. In the yeast, *Saccharomyces cerevisiae*, there are two copies of *hsp90* (HSC82, expressed constitutively and HSP82, heat inducible).

Cells with inactivation of either *hsp82* or *hsc82* grew well at 25°C, but had a growth disadvantage at higher temperatures. Double mutants of the two *hsp90* copies were lethal even at normal growth temperature [3]. In the cyanobacterium, *Synechococcus* sp. PCC 7942, and *E. coli*, *htpG* null mutants had reduced basal and acquired thermotolerance [1, 18], although in *Bacillus subtilis htpG* was not involved in thermotolerance [22]. In *Synechocystis* PCC 6803, *htpG* was shown to be induced by salt stress and hyperosmotic stress [6], although the response to heat stress was not examined. The *in vivo* function of *htpG* in cells is unknown.

Among the heat shock proteins studied thus far, the contributions to thermal protection vary. *In vivo* analysis showed that SmHSP18.1 from *Pisum sativum* has a greater protective role in preventing firefly luciferase (Luc) thermal aggregation than either Hsc70 (constitutive heat shock protein 70) or DnaK [8]. *HtpG* single null mutants (*ΔhtpG*) and double null mutants (*ΔhtpG::hsp16.6*) were obtained to gain an understanding of the *in vivo* role of *htpG* in *Synechocystis*. We demonstrate that *htpG* is necessary for thermotolerance, although *Δhsp16.6* is more sensitive to heat stress than *ΔhtpG*.

Materials and methods

Organisms

Synechocystis sp. PCC (Pasteur Culture Collection) 6803 cells were cultured in BG-11 medium at 30°C under constant fluorescent illumination (40 μE.m^{-2}.sec^{-1}). *Synechocystis* mutants were cultured with chloramphenicol at a final concentration of 5 μg/ml in liquid culture and plates, or kanamycin at a final concentration of 10 μg/ml in liquid culture, and 5 μg/ml in plates [23]. *E. coli* JM109 was a host for molecular manipulation.

HtpG cloning and mutagenesis

The *htpG* gene sequence was obtained from Cyanobase (http://www.kazusa.or.jp/cyanobase/). Primers, forward, 5'-AGACCTAGTGCAGTACTCGAA-3', and reverse, 5'-GCCTCCGCATGATAACTCCTT-3', were used to amplify a 2472 bp fragment, including the 1974 bp *htpG* coding sequence. Primers (forward, 5'-GACCCGGGTTGA CCTGATAGTTTGGCTGT-3', with an extra *Sma* I restriction site at underlined sequence; reverse, 5'- TGCTGCCTGTGATCAAT TCGG-3', with an extra *Kpn* I site at underlined sequence) were used to amplify the 1096 bp chloramphenicol resistance cassette (Cm^r cassette) using plasmid pPZP111 as a template [4].

The 2472 bp PCR product, including the *htpG* gene coding sequence, had a unique *Kpn*I site at 1085 bp and *Sma* I site at 1479 bp. The PCR product was ligated into pGEM-T vector (Promega), designated as pGEMT-htpG, and digested with *Kpn* I and *Sma* I to cut out a 395 bp fragment. The Cm^r cassette was digested with *Kpn* I and *Sma* I enzymes, and inserted into the linearized pGEMT-htpG (Fig. 1).

The transformation was conducted according to Williams [23]. The construct, designated as pHtpG-cat, was transformed into wild type *Synechocystis* and *Δhsp16.6* cells. To confirm that *htpG* was inactivated, genomic DNA was isolated from *Synechocystis* cells using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) and used as a template for PCR.

Absorption measurements

Cell growth rates at 30°C, 37°C, 40°C and 42°C under constant fluorescent illumination (40 $\mu E.m^{-2}.sec^{-1}$) were measured at absorbance 730 nm. Growth rates were measured in triplicate at each temperature.

Measurement of basal and acquired thermotolerance

Cells with a pretreatment at 42°C were exposed for 30 minutes at 50°C under light, and transferred to 30°C to measure growth rate, or serially diluted and spotted onto plates to measure cell survival [10].

Oxygen evolution

Two ml of cells were transferred to the chamber to measure oxygen evolution rate with a Clark-type oxygen electrode (Hansatech, U.K.). $NaHCO_3$, for the CO_2 source, was added to the chamber to a final concentration of 1 mM. All measurements were performed right after treatment in a water circulation chamber maintained at 30°C.

Absorption spectra measurements

Cells were treated at 50°C for 30 minutes under light. After heat treatment, one ml of cells was placed in a cuvette for measurements [11].

Results

HtpG mutations

Northern hybridization detected *htpG* gene expression at normal growth temperature and after heat shock. The size of *htpG* mRNA was approximately 2.2 kb. RT-PCR confirmed that the *htpG* gene is heat inducible (data not shown). To help elucidate the function of HtpG, a Cm^r cassette was inserted to inactivate *htpG* (Fig. 1A). PCR confirmed that mutagenesis was successful (data not shown). RT-PCR was performed to examine *htpG* expression (Fig. 1B). Lanes 3 and 5 showed RT-PCR results using total RNA isolated from *ΔhtpG* and *ΔhtpG::hsp16.6* cells, respectively. No *htpG* transcription product was observed in *ΔhtpG* and *ΔhtpG::hsp16.6* cells. This was in contrast to wild type and *Δhsp16.6* cells (lanes 2 and 4, respectively). Thus, the *htpG* knockout was successful.

Growth rates of mutants

To test the effect of gene mutation on cell growth, we measured cell optical density (OD_{730nm}). Cell growth rates of *ΔhtpG* and *Δhsp16.6* did not differ significantly from the wild type, and the double gene mutation did not affect cell growth at normal temperatures (30°C) (Fig. 2A). At 30°C, wild type, *ΔhtpG*, and *Δhsp16.6* cells were blue green, while *ΔhtpG::hsp16.6* cultures appeared yellow-green. When cells were grown at 37°C, cell growth rate decreased for all cell types (data not shown). At 40°C, wild type cells grew faster than mutant cells (Fig. 2B).

Basal and acquired thermotolerance in *ΔhtpG* and *ΔhtpG::hsp16.6* cells

To test basal thermotolerance, we heat shocked cells without a heat pretreatment (Fig. 2C). After a direct heat shock at 50°C for 30 minutes, wild type cells showed a slow

increase in cell density and after 120 hours began logarithmic growth. The mutant, *ΔhtpG,* showed a similar pattern, but at a slower rate. Mutants, *Δhsp16.6* and *ΔhtpG::hsp16.6*, did not increase in cell number (Fig. 2C). Colony survival of wild type was higher than mutants after heat shock at 50°C for 30 minutes, with 2% of wild type colonies formed, while 0.03% and 0.01% of *ΔhtpG* and *Δhsp16.6* of the colonies formed, respectively. *ΔhtpG::hsp16.6* did not form colonies (Fig. 3A). After heat shock at 50°C for 30 minutes, wild type cells remained green in color. Cells of *ΔhtpG* and *Δhsp16.6* turned yellow-green, while *ΔhtpG::hsp16.6* cells became yellow and died. The absorption spectra of mutants did not change at 30°C; however, after heat shock at 50°C, the absorbance at 625nm decreased for *ΔhtpG*, *Δhsp16.6*, and *ΔhtpG::hsp16.6* cells (data not shown). Absorption at 450 and 680 nm did not decrease during heat shock.

The *ΔhtpG* and *ΔhtpG::hsp16.6* mutants were examined to determine whether acquired thermotolerance was reduced. During pretreatment at 42°C for 60 minutes, wild type, *ΔhtpG,* and *Δhsp16.6* cells were still suspended in the medium; however *ΔhtpG::hsp16.6* cells aggregated and precipitated at the bottom of the flask when cells were placed in a water bath without shaking. When the response to pretreatment at 42°C between wild type and mutants is compared (Figs. 2D and 3B), the pretreatment dramatically increased cell growth and colony survival of wild type, but not mutants. After heat shock at 50°C for 30 minutes, the growth rate of pretreated wild type cells (Fig. 2D) was higher than wild type cells without a pretreatment (Fig. 2C); however, pretreatment did not increase cell growth of *ΔhtpG*, *Δhsp16.6*, and *ΔhtpG::hsp16.6* mutants. Colony survival of wild type increased dramatically from 2% (Fig 3A, without

pretreatment) to 54% (Fig 3B, with pretreatment); however, the pretreatment had little effect on colony survival of *ΔhtpG, Δhsp16.6*, and *ΔhtpG::hsp16.6* with only 0.06%, 0.06% and 0.0005% colony survival with pretreatment, respectively (Fig. 3B), compared to 0.03%, 0.01%, and 0%, respectively (Fig. 3A, without pretreatment).

Oxygen evolution in mutants

At 30°C, oxygen evolution of wild type cells was not significantly different from the three mutant cell types (data not shown). After heat shock at 49°C for 30 minutes, wild type, *ΔhtpG,* and *Δhsp16.6* had a relative oxygen evolution rate of 15%, 8%, and 7%, respectively; however, oxygen evolution was not detected in *ΔhtpG::hsp16.6* cells. After a direct heat shock at 50°C for 30 minutes, the rate of oxygen evolution of wild type was 9% of the control, but 0% in the three mutants (Fig. 3C). When cells were pretreated at 42°C for 60 minutes, then heat shocked at 50°C for 30 minutes, the rate of oxygen evolution of wild type increased from 9% (Fig. 3C, without pretreatment) to 50% (Fig. 3D), although the rate of oxygen evolution in mutant types did not increase. No oxygen evolution was observed in *ΔhtpG, Δhsp16.6,* and *ΔhtpG::hsp16.6* cells (Fig. 3D).

Discussion

From *Synechocystis* genomic DNA, we cloned the *htpG* gene, and demonstrated that it was a heat responsive gene. Northern hybridization analysis showed that the transcriptional level of *htpG* gene was about 1.2 times higher after heat shock. The coding sequence of *htpG* is 1972 bp as determined from the Cyanobase website, and the size of *htpG* mRNA was determined to be about 2.2 kb. Considering the size of the *htpG* coding region and the adjacent reading frames in the genome, it is unlikely that *htpG* is an operon. The *htpG* gene was identified in *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942. One *htpG* ORF was identified in *Anabaena* PCC 7120 by alignment to *Synechocystis* and *Synechococcus htpG* (data not shown). Amino acid identity between *Synechocystis* 6803 and *Anabaena* PCC 7120 is 68%, 63% between *Synechocystis* 6803 and *Synechococcus* PCC 7942, and 67% between *Synechococcus* PCC 7942 and *Anabaena* PCC 7120.

The effects of *htpG* inactivation on cell growth vary among prokaryotes. In *E. coli* [1], *Actinobacillus actinomycetemcomitans* [24], and *Synechococcus* sp. PCC 7942 [18], the *htpG* mutation had little effect on cell growth at optimal growth temperature, but as culture temperature increased, the rate of growth decreased. *Hsp90* was essential for growth of the yeast, *Saccharomyces cerevisiae*, at all temperatures tested [3]. In contrast, the growth rate of the *htpG* null mutant of *Bacillus subtilis* was unaffected at both optimal growth and elevated temperatures [22]. Our results showed that *Synechocystis* mutants were not affected at 30°C and 37°C, but showed little to no growth at 40°C. This suggests that HtpG in *Synechocystis* is not essential for cell growth at optimal growth temperature.

Some heat shock proteins play roles in basal and acquired thermotolerance. HtpG is essential for thermotolerance in *Synechococcus* PCC 7942 [18] and *E. coli* [1, 20]. However, the deletion of *htpG* did not affect thermotolerance in *Bacillus subtilis* [22]. Inactivation of *smhsp* (*IbpA/B*) in *E coli* also results in loss of basal and acquired thermotolerance [7]. Overexpression in *E. coli* of four *smhsp* genes: *smhsp* from the brine shrimp, *Artemia franciscana*, *oshsp* from *Oryza sativa* cytoplasm, *tom111* from *Lycopersicon esculentum* chloroplasts, and *hsp16.6* from *Synechocystis* sp. PCC 6803, conferred thermal protection during heat stress [15]. Our results showed that a direct heat shock decreased wild type and mutant cell growth in culture and on plates. A heat pretreatment did not significantly increase acquired thermotolerance in mutants, indicating that *ΔhtpG, Δhsp16.6,* and *ΔhtpG::hsp16.6* lost both basal and acquired thermotolerance.

Oxygen electrode measurement indicated that oxygen evolution was adversely affected during heat shock. Absorption spectra measurements demonstrated that the ability to absorb light at 625 nm was reduced in mutant cells, the absorbance peak of phycocyanin from phycobilisomes (PBS). In cyanobacteria and red algae, phycobilisomes are primary light-harvesting antennae for photosystem II, located on the stromal side of the thylakoid membrane, and are primarily composed of brilliantly color family of phycobiliproteins [17]. After a heat shock at 50°C of pretreated cells, mutant cells turned yellow, oxygen evolution also was lost (data not shown). These results showed that the inactivation of either *htpG* or *hsp16.6* adversely affected oxygen evolution and possibly the thermal stability of phycobilisomes during heat stress.

The function of each family of HSPs differs in cells and may be reflected in the ability of a specific HSPs to protect cells against heat stress [8]. We compared the protective roles of *htpG* and *hsp16.6* to heat stress using single mutants and double mutants. We were able to demonstrate that cells became more sensitive to elevated temperatures when *hsp16.6* was inactivated and was lethal when both *hsp16.6* and *htpG* were inactivated in cells. This suggests that the protective ability of *htpG* and *hsp16.6* during heat shock was different, and that both genes play a role in protecting cells from elevated temperatures. *In vitro* analysis indicated that, during heat stress, HSP16.6 binds to denatured proteins and delivers them to GroEL-GroES and DnaK-DnaJ-GrpE chaperone systems for renaturing, and then maintains the proteins in an active form [21]. The comparison of *in vitro* roles between HSP16.6 and HtpG is necessary to further understand why HSP16.6 had more *in vivo* protective roles than HtpG. Moreover, to gain further information about the *in vivo* role of HSPs in *Synechocystis* cells, multiple mutations need to be constructed to study the effects. This work is an initial study on the *in vivo* functional role comparison of heat shock proteins in cyanobacteria.

Acknowledgements

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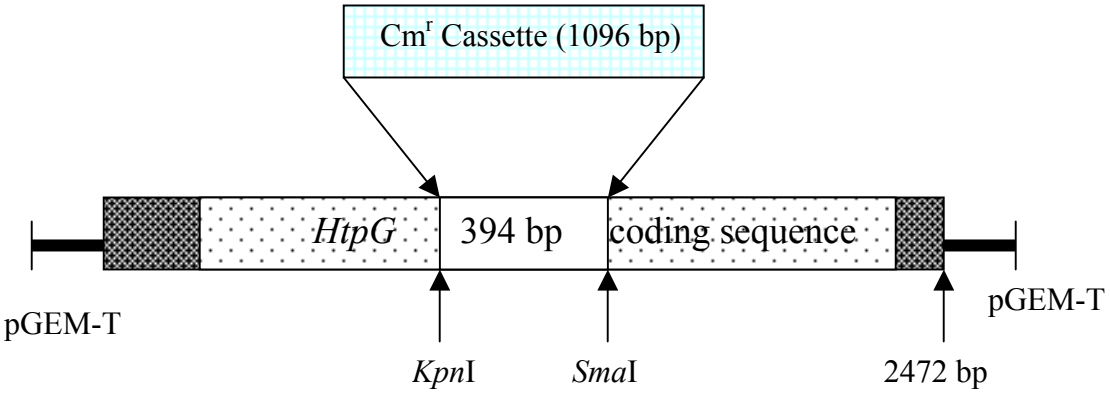
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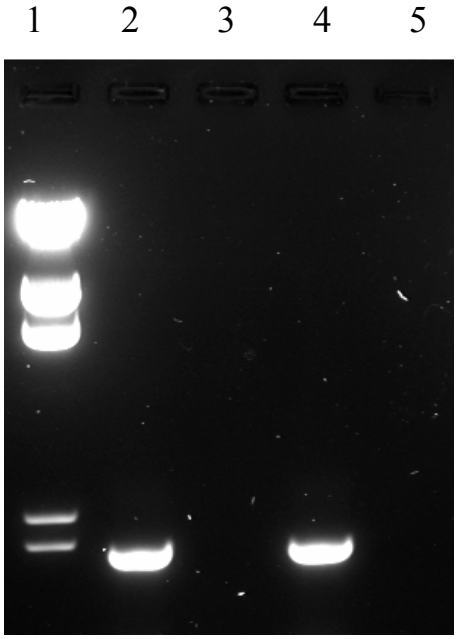
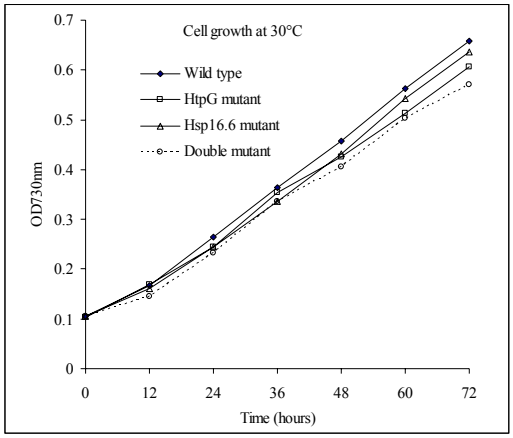
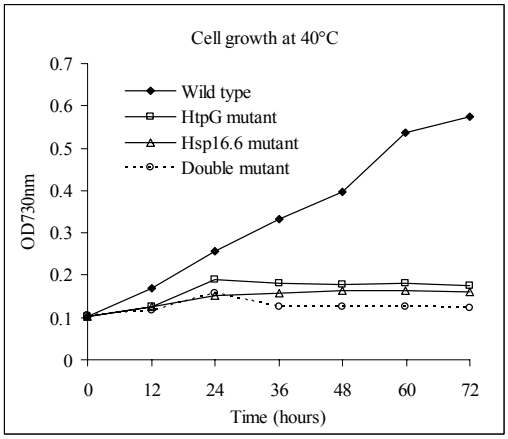

1
2
3
4
5

Fig. 1. The *htpG* null mutant was constructed by insertion of a chloramphenicol resistance cassette (Cm^r) (A). Confirmation of the *htpG* gene null mutation by RT-PCR (B). Lane 2, wild type, lane 3, *ΔhtpG,* lane 4*, Δhsp16.6,* and lane 5, *ΔhtpG::hsp16.6*. Lambda DNA cut with *Hin*d III as the DNA marker (lane 1). The figure is not drawn to scale.

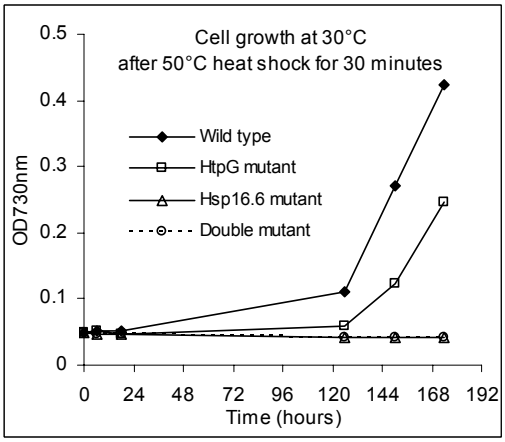
A


Cell growth at 30°C
Wild type
HtpG mutant
Hsp16.6 mutant
Double mutant
OD730nm
Time (hours)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
12
24
36
48
60
72

B


Cell growth at 40°C
Wild type
HtpG mutant
Hsp16.6 mutant
Double mutant
OD730nm
Time (hours)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
12
24
36
48
60
72

C


Cell growth at 30°C
after 50°C heat shock for 30 minutes
Wild type
HtpG mutant
Hsp16.6 mutant
Double mutant
OD730nm
Time (hours)
0
0.1
0.2
0.3
0.4
0.5
0
24
48
72
96
120
144
168
192

D

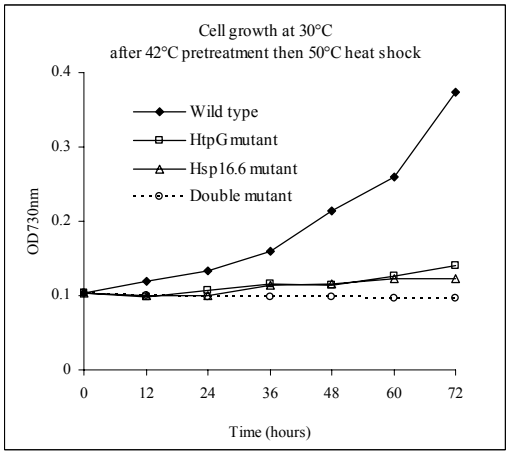
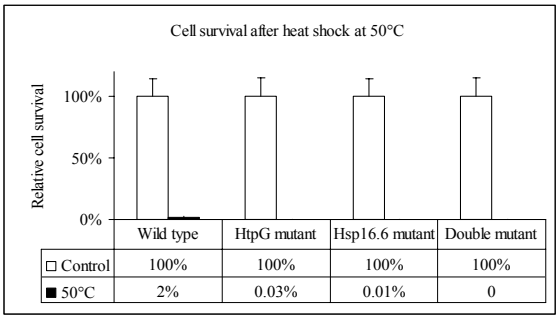

Cell growth at 30°C
after 42°C pretreatment then 50°C heat shock
Wild type
HtpG mutant
Hsp16.6 mutant
Double mutant
OD730nm
Time (hours)
0
0.1
0.2
0.3
0.4
0
12
24
36
48
60
72

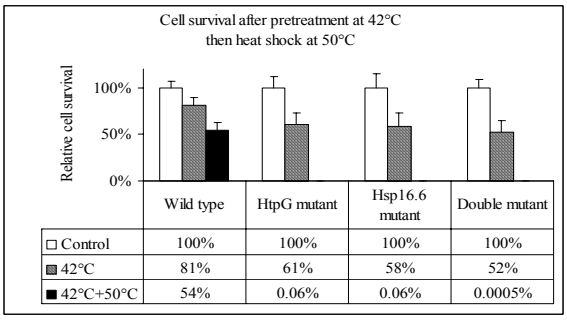
Fig. 2. Cell growth of wild type, *ΔhtpG, Δhsp16.6,* and *ΔhtpG::hsp16.6*. Cell growth at 30˚C (A), cell growth at 40˚C (B), cell growth at 30˚C after heat shock at 50°C for 30 minutes (C), and cell growth at 30˚C after pretreatment at 42˚C for 60 minutes then heat shock at 50°C for 30 minutes (D).

A



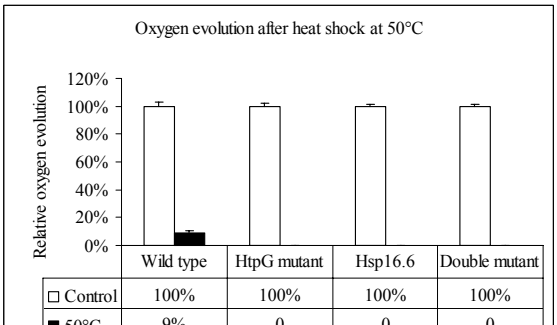
	Wild type	HtpG mutant	Hsp16.6 mutant	Double mutant
□ Control	100%	100%	100%	100%
■ 50°C	2%	0.03%	0.01%	0

B



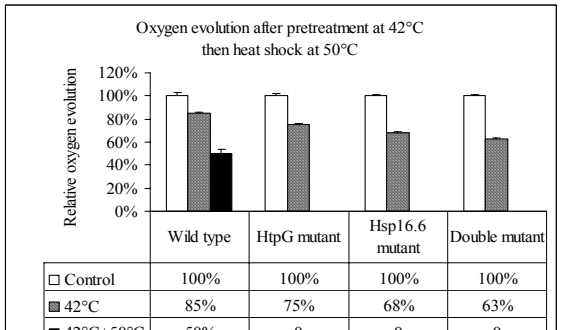
	Wild type	HtpG mutant	Hsp16.6 mutant	Double mutant
□ Control	100%	100%	100%	100%
▨ 42°C	81%	61%	58%	52%
■ 42°C+50°C	54%	0.06%	0.06%	0.0005%

C



	Wild type	HtpG mutant	Hsp16.6	Double mutant
□ Control	100%	100%	100%	100%
■ 50°C	9%	0	0	0

D



	Wild type	HtpG mutant	Hsp16.6 mutant	Double mutant
□ Control	100%	100%	100%	100%
▨ 42°C	85%	75%	68%	63%
■ 42°C+50°C	50%	0	0	0

Fig. 3. Comparison of cell survival and oxygen evolution after heat shock. Cell survival after heat shock at 50°C for 30 minutes (A), cell survival after pretreatment at 42˚C for 60 minutes and then heat shock at 50°C for 30 minutes (B), oxygen evolution after heat shock at 50°C for 30 minutes (C), and oxygen evolution after pretreatment at 42˚C and then heat shock at 50°C for 30 minutes (D).

Appendix

This appendix contains experimental data and results not included in the two manuscripts (Chapters 2 and 3).

Hsp-related ORFs in Synechocystis cells

Since the whole Synechocystis sp. PCC 6803 genomic sequence has been completed, we have searched four heat shock genes related ORFs from the genome database (http://www.kazusa.or.jp/cyanobase/) (Fig. 1). The ORFs were PCR amplified (Table 1). Hsp33 ORF was PCR amplified by two primers (forward, 5'-ATGGCAGACC AATTGATCCGC-3', and reverse, 5'-GCTTTTCGGCTTTTTCCC-3'). HtpG ORF was PCR amplified with primers (forward, 5'- AGACCTAGTGCAGTACTCGAA -3', and reverse, 5'-GGGTTAATACCGCATTAGAG-3'). The PCR product was used as a probe for Northern hybridization.

Northern hybridization detected htpG gene expression at normal growth temperature and after heat shock (Fig. 2). The htpG induction level after heat shock (lane 2) was slightly (1.2 fold) higher than non-heat shock condition (lane 1). RT-PCR confirmed that the htpG gene was an inducible gene (data not shown). Northern hybridization also showed that hsp16.6 gene increased dramatically after heat shock [6]. However, sll1061 (smhsp homolog) and sll1988 (hsp33 homolog in E. coli cells) expression could not be detected by Northern hybridization. This suggested that the expression levels of sll1061 and sll1988 may be too low to be detected by Northern hybridization, or may not have expression in cells.

The htpG genes were identified in Synechocystis PCC 6803 [2] and Synechococcus PCC 7942 [13], respectively. We searched Cyanobase and found one

htpG ORF each from Anabaena PCC 7120 and Thermoynechococcus elongatus BP-1 (http://www.kazusa.or.jp/cyano/cyano.html). The alignment of htpG showed that nucleic acid similarity is high among the four cyanobacteria, from 65% to 68% (data not shown), and amino acid sequence identity is also very high, from 63% to 69% (Fig 3). However, HtpG amino acid sequence identity between cyanobacteria and other eubacteria is less than 40% (Table 2).

Mutagenesis of htpG in Synechocystis cells

The *htpG* gene was mutated to elucidate the function of HtpG *in vivo*. PCR was performed to amplify the *htpG* and *hsp16.6* fragment using genomic DNA as a template to screen the *htpG* mutant and double mutant Δ*hsp16.6::htpG* (Fig. 4). The *htpG* PCR product using the mutated DNA as a template would be about 3.2 kb, and the *hsp16.6* PCR product using the mutated DNA as a template would be 3.5 kb [6]. The *htpG* and *hsp16.6* PCR product for wild type is 2.5 kb (lane 1) and 2.6 kb (lane 2), respectively; 3.2 kb (lane 3) and 2.6 kb (lane 4) in *ΔhtpG*, 2.5 kb (lane 5) and 3.5 kb (lane 6) in *Δhsp16.6,* and 3.2 kb (lane 7) and 3.5 kb (lane 8) in *ΔhtpG::hsp16.6*, respectively. Table 3 shows *E. coli* strains and plasmids used in this study and chapter 3.

The effect of *htpG* mutation on cell growth

Synechocystis wild type and mutant cells were grown in BG-11 medium at 30°C, 37°C, 40°C, and 42°C under constant fluorescent illumination (40 μE/m^2.s). At 30°C (result in Chapter 3), cell growth of wild type and mutant cells showed little difference. At 37°C, absorbance at 730nm for cell growth and 625nm for phycobilisomes in wild type and mutant cells showed little difference (Fig 5A &B). Mutant cells showed a

growth defect at 40°C (result in Chapter 3). At 42°C, cell growth was not observed in wild type and mutant cells, and cells turned yellow after 24 hours (Fig 5C).

Absorbance at 625 nm decreased in mutants under heat stress condition

We observed cell color changed during heat stress in the culture flasks and by bright-field light microscopy. Mutant cells turned yellow, while wild type cells remained green. Cells were grown in BG-11 medium, and aliquoted into eight culture tubes before heat shock treatment. Cells remained green without heat shock (Fig 6, control tubes). After treatment at 50°C for 30 minutes, wild type cells remained green, but *ΔhtpG,* and *Δhsp16.6* cells became a little yellow, and the double mutation *ΔhtpG::hsp16.6* became yellow (Fig. 6, heat shock tubes). Longer treatment (up to one hour) at 50°C resulted in the bleaching of *ΔhtpG::hsp16.6* cells (data not shown). Cells were also heat shocked and absorption spectra were measured. The results showed that the absorbance of mutants did not change at growth temperature (Fig. 7A), which suggested that *htpG* and *hsp16.6* mutation did not affect absorbance spectra. After heat shock, absorbance at wavelength 625nm decreased in *ΔhtpG, Δhsp16.6*, and *ΔhtpG::hsp16.6* cells (Fig. 7B).

The detection of *hsp16.6* in cyanobacteria

Since *smhsps* have been cloned from two cyanobacterium species [11, 2], Southern hybridization was conducted using the *hsp16.6* coding sequence as a probe to detect additional *smhsps* in other cyanobacteria. Five μg of genomic DNA isolated from cyanobacteria (kindly provided by Brian Henson in the lab) was digested with *Eco*R I. The digested genomic DNA was run on agarose gel, and transferred to nylon membrane. Chemical labeling and detection kit (Amershan) was used for Southern hybridization. The results did not show any bands for cyanobacteria (*Nostoc* PCC 6720, *Nostoc* PCC

7120, *Nodularia* PCC 7804, *Cylindrospermum* PCC 7417, *Chlorogloeopsis* PCC 6912, *Scytonema* PCC 7110, *Fischerella* UTEX 1903, *Calothrix* ATCC 27914, *Anabaena* ATCC 33047). The lack of signal may due to the weak hybridization ability for low similarity of sequence homology or the low sensitivity of chemical labeling and detection system (data not shown).

Cold shock in *hsp16.6* mutant

The function and induction of *hsp16.6* during cold stress was studied, since some heat shock genes have general function during stress. The *htpG* mutation adversely affected thermotolerance and cold resistance in *Synechococcus* sp. PCC 7942 [5]. *Synechococcus hsp100* (*clpB*) was induced by heat shock and low temperature, and the *clpB* null mutation was sensitive to heat shock and low temperature [1, 10]. Since HSP16.6 plays a role in thermotolerance, to test whether HSP16.6 can protect cells during low temperatures, wild type and *Δhsp16.6* cells were treated at 15°C for three hours. Wild type retained 92% of oxygen evolution activity compared to non-cold treatment (control), and *Δhsp16.6* cells retained 84% of activity (Fig. 8A). Western blotting showed that cold shock did not induce HSP16.6 protein (See Chapter 2, Fig 1B). The possible explanation is that, although HSP16.6 expression level is very low at normal growth conditions, HSP16.6 is integrated into the thylakoid membrane [4], so the deletion of HSP16.6 might affect the integrity of thylakoid membrane, therefore decreases cold resistance.

Shuttle reporter system

The shuttle vector used in Chapter 2 was from RSF1010 derivative pKT210, and pL526D. RSF1010 is a low-copy-number (12-14 copies per cell), broad-host-range

plasmid [12]. This promiscuous IncQ plasmid has *oriV*, and can replicate in a variety of prokaryotes hosts, which offers a simple and alternative method for gene transformation [12]. The RSF1010 accession number from NCBI (http://www.ncbi.nlm.nih.gov/) is M28829. The RSF1010 total sequence is 8684 base pairs. The *Xmn*I site is at 2034, and *Pst*I site is 7772, and 8572. From the RSF1010 complete sequence, the element and their position in the RSF1010 is as follows: Sm resistance protein A (63 to 866), Sm resistance protein B (866 to 1702), *oriV* (2347 to 2742), mobilization protein C (2767 to 3051), mobilization protein A (3250 to 5379), mobilization protein B (3998 to 4411), replication protein B (4408 to 5379, unknown protein E (5440 to 5652), repressor protein F (5654 to 5860), replication protein A (5890 to 6729), replication protein C (6716 to 7567), Sm resistance protein (7875 to 8663) [12].

Some unique and poly cloning sites were added to the shuttle vector (for Cm^r gene, *Kpn*I, *Eco*O109I, and *Cla*I restriction sites at the one end, *Pst*I and *Csp*45I at the other end), so the vector was convenient for molecular manipulation. The triparental conjugation is dependent on *E. coli* Cm404, which harbors the helper plasmid pRK2013 [9]. In this study, we constructed a conditional expression vector controlled by the heat shock *hsp16.6* promoter, which can be used for cyanobacteria [7, 8]. It can also be used to study the *in vivo* function of cyanobacteral genes.

Acknowledgements

We thank Dr. Sengyong Lee for the *hsp16.6* and *sll1061* sequence alignment, Brian Henson to provide cyanobacteral genomic DNA, and Dan Prochaska for the *hsp33* sequence alignment.

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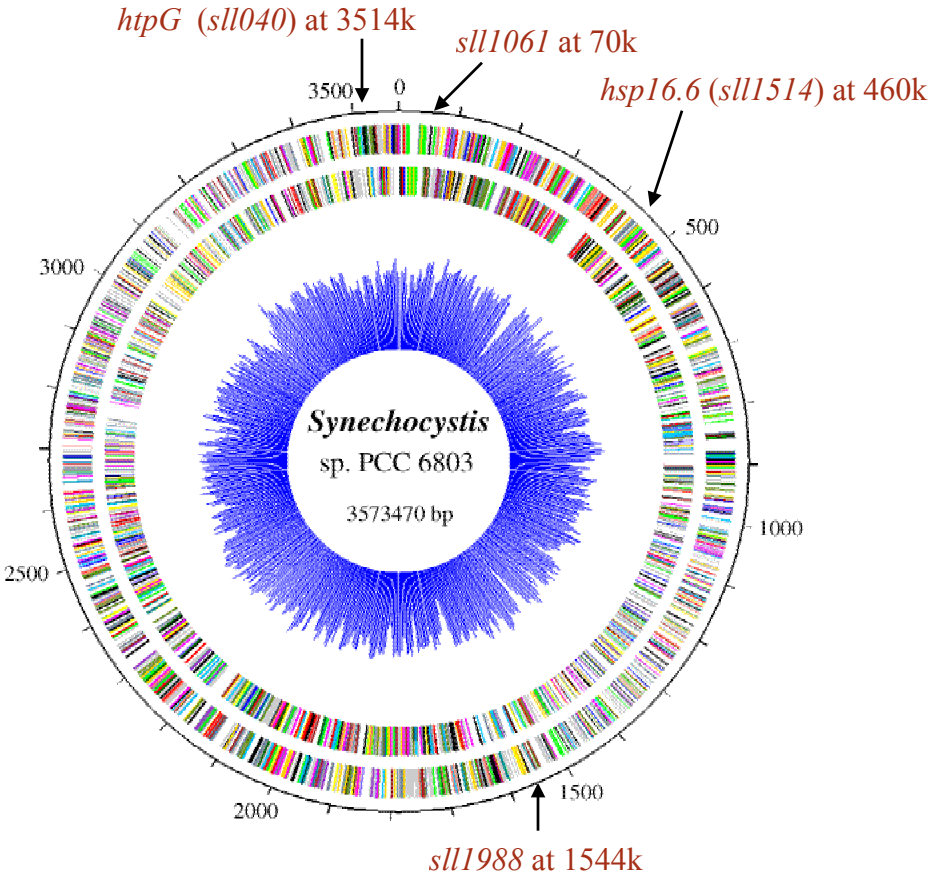

htpG (*sll040*) at 3514k
sll1061 at 70k
hsp16.6 (*sll1514*) at 460k
sll1988 at 1544k
3500
0
500
1000
1500
2000
2500
3000
Synechocystis
sp. PCC 6803
3573470 bp

Fig. 1. The positions of heat shock-related ORFs in the *Synechocystis* genome.

1 2

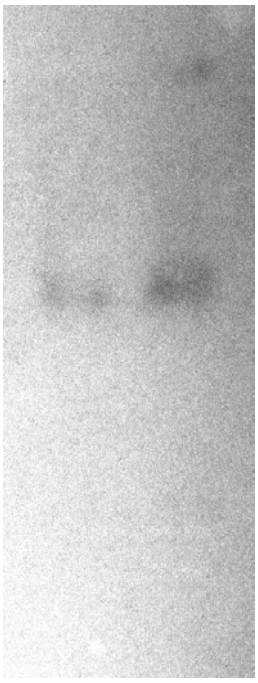
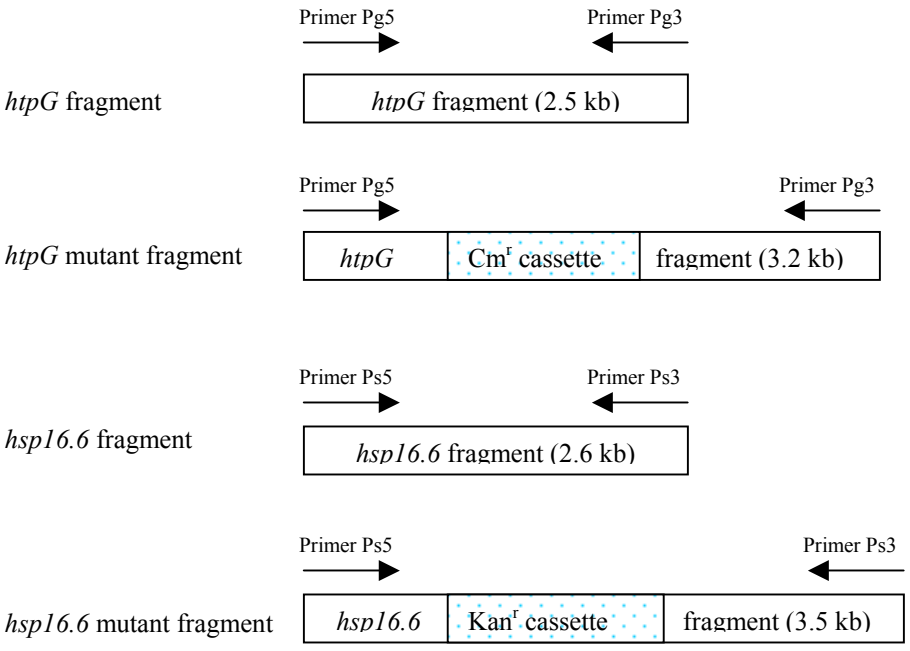


Fig. 2. Northern hybridyzation of *htpG* gene in *Synechocystis* cells. Cells were treated at 42°C for one hour. 30 μg of RNase-free DNAse treated RNA was electrophoresed on a 1% formaldehyde denaturing agarose gel, and transferred onto a nylon membrane. The *htpG* coding sequence of the PCR product was used as a probe for hybridization. The lanes are: cells grown at 30°C as control (lane 1) and heat shock at 42°C (lane 2).

```
Anabaena          --MLEQGTISIHTENIFPIIKKSLYSDHQIFLRELVSNAVDAIQKLKMVSRAGEYAGVVD
Thermo            --MLEQGTISIHTENIFPIIKKWLYSDHEIFLRELVSNAVDAIQKLRMVARSGEYSGDVD
Synechocystis     MAVLEKGNITIHTENIFPIIKKSLYTDHEIFLRELISNAVDAISKRKMAAFASDGSGEVP
Synechococcus     MAILEQGNITIHTDNIFPIIKKSLYSEHEIFLRELISNAVDAIQKLKMVSYAGELEGEIG
                    :**:*.*:***:******** **::*:******:*******.* :*.: :.:  * :

Anabaena          EPEIQLAIDKDKKTLSITDNGIGMTAEEVKKYINQVAFSSAEEFIHKYQGKSDQPIIGHF
Thermo            HPEVTITIDKENKKLAIADNGIGMTAEEVKKYITQVAFSSAEEFVQKYKGEGENAIIGHF
Synechocystis     DPQITLVVDKVNKTLSITDNGIGMTADEVKKYINQVAFSSAEEFIEKFQKSAN-DLIGHF
Synechococcus     DPQITLSIDRDRKQLKIADNGIGMTADEIKRYINQVAFSSAEDFIEKYKGGADQPIIGHF
                  .*:: : :*: .* * *:********:*:*:**.********:*:.*::  .:  :****

Anabaena          GLGFYSSFMVAQKVEIDTLSYQEGAQAVHWSCDGSPEFTLEESSRTTIGTTITLTLLPDE
Thermo            GLGFYSAFMVAERVEIDTLSYREGAVPVHWTCDGSTEFTLADGQRTTVGTTVTLTLQDSE
Synechocystis     GLGFYSAFMVSQKVEIDTLSYQEGAQAVHWSCDGSPEFELTDSDRQQVGTTVTLTLLDDE
Synechococcus     GLGFYSAFIVADRVEIETLSYQKGATPVHWTCDGSPSFELSEGGSRTERGTTIILNLSEEE
                  ******:*:*:::***:****::** .***:****..* * :..*   ***: *.*  .*

Anabaena          EEYLESARVKNLVKTYCDFMPVPIKLDGEVLNRQKAPWRESPSNLTKEDYLEFYRYLYPF
Thermo            LEYLEPARIRQLVRKYCDFLPVPIKLEGEQINRQIAPWKSAPNSLSKEDYLEFYRYLYPF
Synechocystis     QEYLETGRIRQLVKTYSDFMAVPIRFEGETLNKQRAIWKESPQNLTKEDYLEFYRYLYPF
Synechococcus     LEYLEPARIRQLVKTYCDFMPVPIALEGEVLNKQKAIWRESAGNLKEEDYQEFYRYLYPF
                   ****..*:::**:.*.**:.*** ::** :*:* * *:.:. .*.:*** *********

Anabaena          QEEPLLWVHLNTDYPFIINGILYFPKMRPDVDVTKGQIKLFCNQVFVSDNCEEIIPQFLV
Thermo            QEDPLLWVHLNTDYPFVVNGILYFPKLRPDIDVTKGQIKLYCNQVFVSDNCEEVIPRFLL
Synechocystis     QEDPLLWVHLNTDYPFLLNGILYFPKLRPDVDVSKGQIKLFCNQVFVSDHCEEVIPEFLM
Synechococcus     QDEPLLWVHLNTDYPYQVNGVLYFPKLRPDIDLTQGQIKLFCNQVFVSDHCEEVVPRFLL
                  *::************: :**:*****:***:*:::*****:********:***::*.**:

Anabaena          PMRGVIDSTDIPLNVSRSALQGDRTVRKIGDYIAKKVGDRLKELYRDDREQYISAWKDLS
Thermo            PLRGVIDSSDIPLNVSRSFLQNDRTVRKIADYIAKKVGDRLKELYREDPAAYVRLWQDLG
Synechocystis     PLRGVIDSPDIPLNVSRSALTNHRTVRRIADYIAKKVGDRLKSLYDENPAEYLKCWEDIG
Synechococcus     PMRGVIDSPDIPLNVSRSALQADRKVRSIAGFIAKKVGDRLKELYRSQRDQYLNIWSDLS
                  *:******.********* *  .*.** *..:**********.** .:   *:  *.*:.

Anabaena          TFVKFGVLNDEKFKKQVEDIIIFRSTVKWEQPAETPAVEVQSQEGDVWQDITPSPDSPTP
Thermo            TFVKFGSINDEKFKKQVEDILIYRTTAALTSKERDDVWAAEG-------------GIQVD
Synechocystis     TFVKFGSLREDKFKQQVEDLLLYRTTYKATAESTEPKVEVLTEDGDAWAETNQSDGVNPY
Synechococcus     TFVKFGCLNDQKFREQVKEILVFRSSAELSEATSS-------------------DRGQDS
                  ****** :.::**::**::::::*::

Anabaena          G-LPYTTLKEYLERNKERHENRVFYSTDEATQATYIELHKNQGLEVLFLDSFIDT-HFIN
Thermo            G-KTYTTLKEYLERNKERQGNRVYYCTDEVNQATYVQLLKSQGIEVLFMDSFIDT-HFVP
Synechocystis     EKEGYTSLKRYLERNKERHENRVFYCTDQATQATYVELYKNQGIEVLFMDSFIDTNYFIP
Synechococcus     QGNWYTTLQAYLERNQEKHENRVYYCTDAASQATYIELFRKKGLEVLYLDSFIDS-HFVS
                      **:*: *****:*:: ***:*.** ..****::* :.:*:***::*****: :*:

Anabaena          FLEQEYQDVKFTRVDSDLDNTLLEQDKAGEIVDPTTNKTKSEIIKELFEKSLNKPKVNIR
Thermo            WLEQNYRDVKFLRVDAELDETLIDKSKESELIDPTTNKTRSEQIKALFESVLKKPKLTIR
Synechocystis     FLEKEYSDVKFSRVDSELDQSLIEEDKASDIVDPATNKSRSEQIKEIFEKALNNPNINIK
Synechococcus     LLEQEYQDVRFSRVDAELDDTLLET-EEAEIVDPTTNKTRSQTLKELFEAAIANSRINVR
                   **::* **:* ***::**::*::  : .:::**:***::*: :* :**  : :..:.::

Anabaena          TEALKSDDPQGTPPAIVLLPEFLRRMREMTAMMQQQN-ADFPEDHILLVNTAHPLIQNLV
Thermo            TEALKADAP----PAMILLPEASRRMQEMMAMMQQQANVQLPEEHILVVNTAHPLVQNLL
Synechocystis     TQSLKSDDPQSTPPAMVLLPEAMRRLQEMTAMMQQQA-MAFPEQHVLAINTNHPLIKNIL
Synechococcus     TESLK-DTDASAPPAMVLLPEAARRMREMSAFLGQDP-ANLPDDHVLLVNTAHPLVQNIL
                  *::** *      **::****  **::** *:: *:    :*::*:* :** ***::*::

Anabaena          NLNQGAIIQGDGESTTNPLVNLICQHVYDLALMSQKGFDAEGMKSFVERSNDVLTKLTQQ
Thermo            SLNQGTIIQTSGTSETGTLVEKLCQYIYDLALMSQRAFDANGMQQFTERASQVLTQLTTM
Synechocystis     SLSQGGIVTGSGESPSAELAKSLCQHVYDLALMAQKGFDAEGMKGFIERSNAVLTRLTKQ
Synechococcus     SLQQGAILSSDGHSPSQVLAEQLCRHIYDLALMTQKGFDAEGMKAFIERSNAVLTALTTR
                  .*.** *:  .* * :  *.: :*:::******:*:.***:**: * **:. *** **

Anabaena          AKN
Thermo            ATR
Synechocystis     ---
Synechococcus     Q--
```

Fig. 3. Comparison of the putative amino acid sequence of HtpG in cyanobacteria. *Synechocystis* sp. PCC 6803, NP_443009; *Anabaena* sp. PCC 7120, slr2323 from Cyanobase; *Nostoc* sp. PCC 7120, NP_486363; *Synechococcus* sp. PCC 7942, BAA85851; *Thermosynechococcus elongatus* BP-1 (*Thermo*), tll1191 from Cyanobase.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Primer Pg3
htpG fragment
htpG fragment (2.5 kb)
Primer Pg5
Primer Pg3
htpG mutant fragment
htpG
Cmr cassette
fragment (3.2 kb)
Primer Ps5
Primer Ps3
hsp16.6 fragment
hsp16.6 fragment (2.6 kb)
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Primer Ps3
hsp16.6 mutant fragment
hsp16.6
Kanr cassette
fragment (3.5 kb)

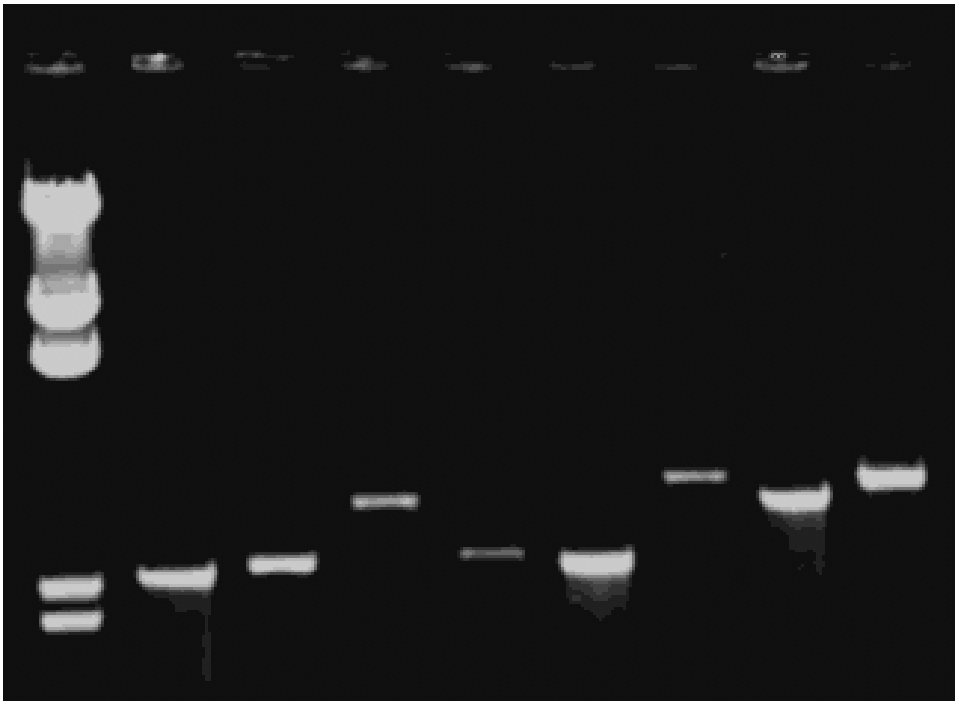
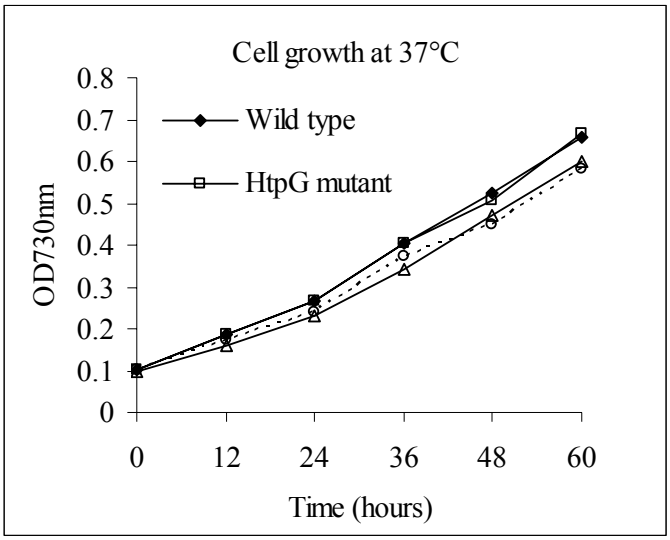
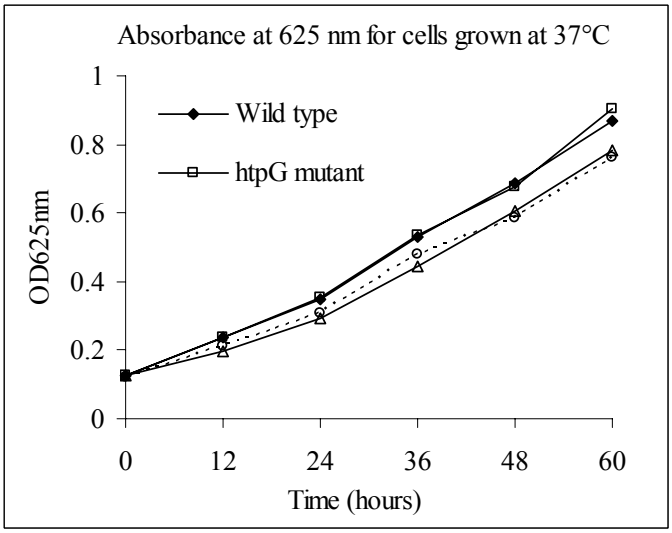


Fig. 4. Confirmation of mutation using PCR. The genomic DNA was isolated from four types of cells. Pg5/3 or Ps5/3 primers was used to PCR amplify *htpG* and *hsp16.6* fragment, using genomic DNA as a template. Lane 1, Pg5/3 primers for wild type, lane 2, Ps5/3 primers for wild type, lane 3, Pg5/3 primers for *ΔhtpG*, lane 4, Ps5/3 primers for *ΔhtpG*, lane 5, Pg5/3 primers for *Δhsp16.6*, lane 6, Ps5/3 primers for *Δhsp16.6*, lane 7, Pg5/3 primers for double mutation *ΔhtpG::hsp16.6*, lane 8, Ps5/3 primers for *ΔhtpG::hsp16.6.* Lambda DNA cut with *Hin*d III was used as the DNA marker (lane M).

A


Cell growth at 37°C
Wild type
HtpG mutant
OD730nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
12
24
36
48
60
Time (hours)

B


Absorbance at 625 nm for cells grown at 37°C
Wild type
htpG mutant
OD625nm
1
0.8
0.6
0.4
0.2
0
0
12
24
36
48
60
Time (hours)

C

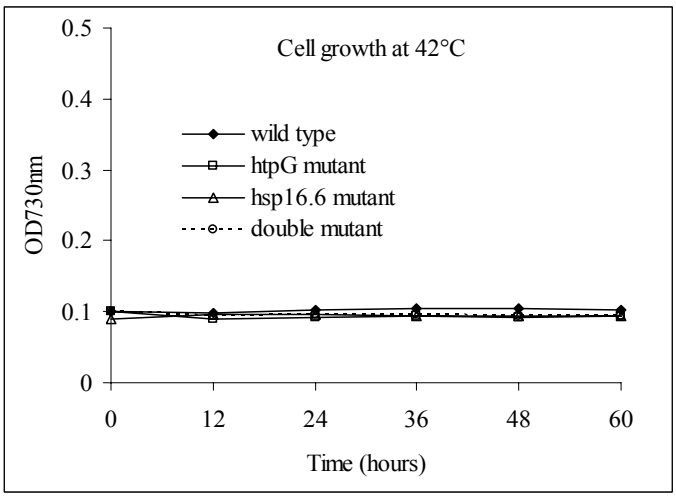

Cell growth at 42°C
wild type
htpG mutant
hsp16.6 mutant
double mutant
OD730nm
0.5
0.4
0.3
0.2
0.1
0
0
12
24
36
48
60
Time (hours)

Fig. 5. Cell growth of wild type, *ΔhtpG, Δhsp16.6,* and *ΔhtpG::hsp16.6*. Growth rates were measured using absorbance at 730 nm, and phycobilisomes were measured at 625nm. Cell growth at 37˚C (A), phycobilisomes at 625nm for cells grown at 37˚C (B), and cell growth at 42˚C (C).

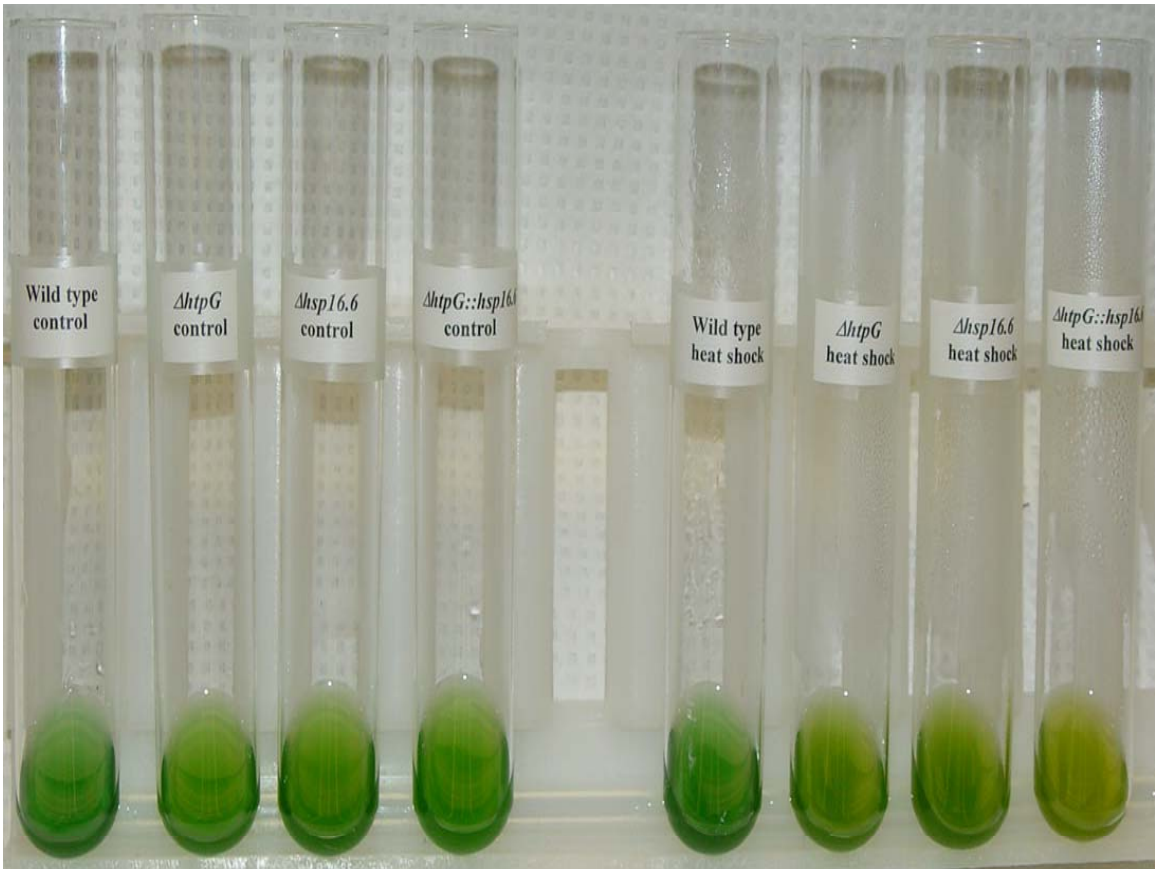
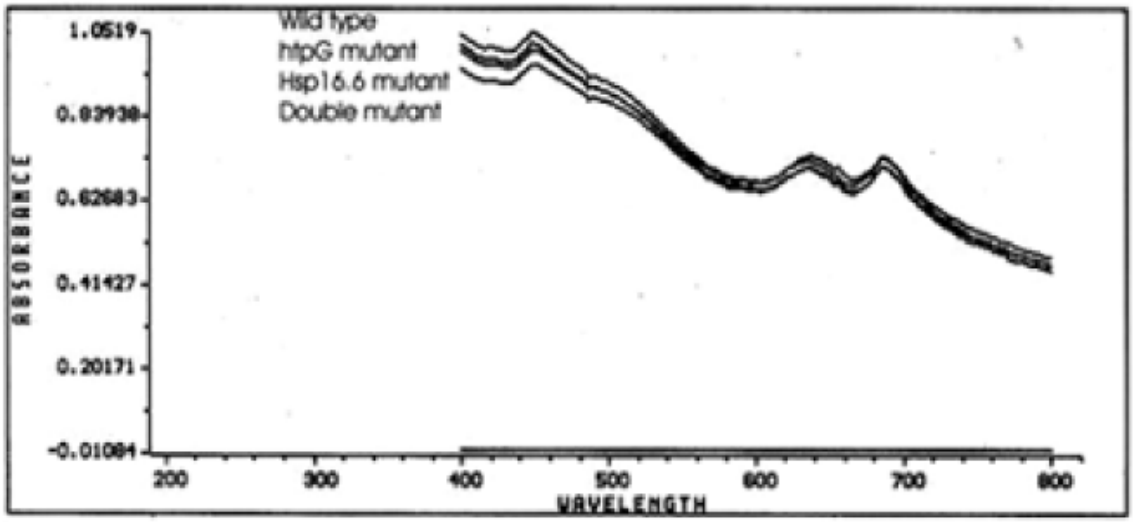

Wild type control
ΔhtpG control
Δhsp16.6 control
ΔhtpG::hsp16.6 control
Wild type heat shock
ΔhtpG heat shock
Δhsp16.6 heat shock
ΔhtpG::hsp16.6 heat shock

Fig. 6. The effect of heat shock at 50˚C for 30 minutes on cell culture color in wild type, *ΔhtpG, Δhsp16.6,* and *ΔhtpG::hsp16.6*.

A


Wild type
htpG mutant
Hsp16.6 mutant
Double mutant
1.0519
0.83938
0.62683
0.41427
0.20171
-0.01084
ABSORBANCE
200
300
400
500
600
700
800
WAVELENGTH

B

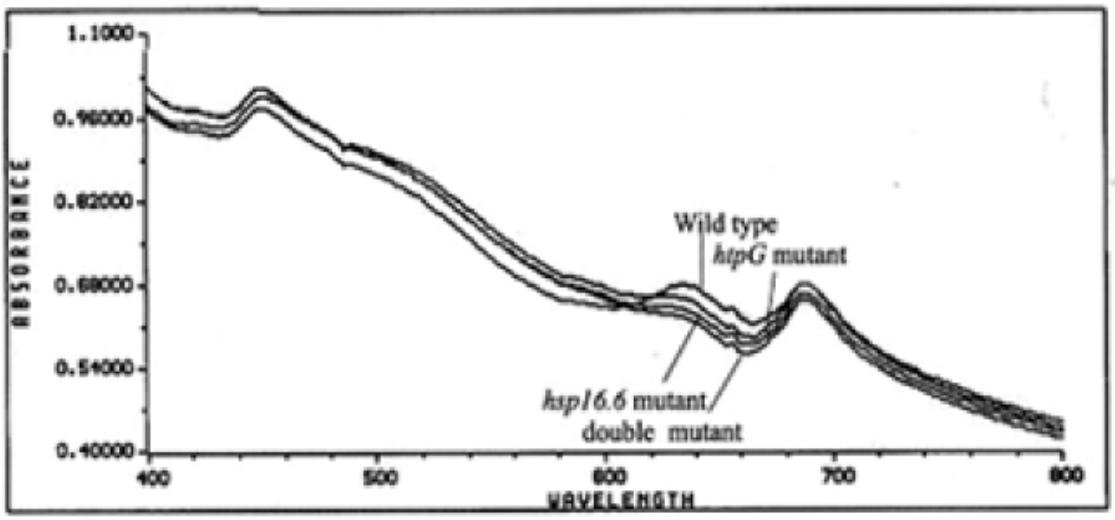

1.1000
0.96000
0.82000
0.68000
0.54000
0.40000
ABSORBANCE
Wild type
htpG mutant
hsp16.6 mutant
double mutant
400
500
600
700
800
WAVELENGTH

Fig. 7. Absorbance of wild type, *ΔhtpG, Δhsp16.6,* and *ΔhtpG::hsp16.6*. without heat shock (A), or treated at 50°C for 30 minutes (B).

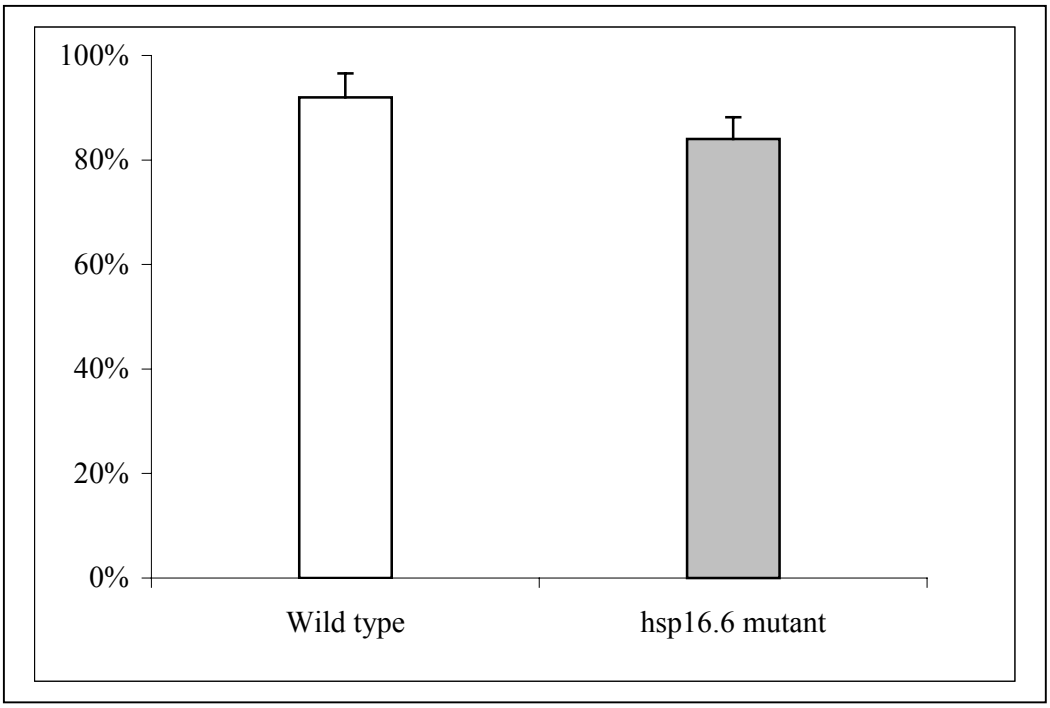

100%
80%
60%
40%
20%
0%
Wild type
hsp16.6 mutant

Fig. 8. The effect of cold stress on wild type and *hsp16.6* mutant cells. Cells were treated at 15°C for three hours, and oxygen evolution was measured at 30ºC.

Table 1. Hypothetical heat shock genes in *Synechocystis* cells.

Genes	Cyanobase Keyword	Initiation base in the genome	Termination base in the genome	Amino acid length	Expression in cells
hsp16.6	Sll1514	460690	460250	146	Yes
htpG	Sll0430	3516208	3514232	658	Yes
smhsp putative gene	Sll1061	70935	70420	171	No
hsp33 homolog	Sll1988	1544665	1543757	302	No

Table 2. Comparison of the putative amino acid sequence of HtpG in cyanobacteria and other prokaryotes. *Synechocystis* sp. PCC 6803 (S6803), NP443009; *Anabaena* sp. PCC 7120, slr2323 from Cyanobase; *Nostoc* sp. PCC 7120, NP486363; *Synechococcus* sp. PCC 7942 (S7942), BAA85851; *Thermosynechococcus elongatus* BP-1 (Thermo), tll1191 from Cyanobase; *Actinobacillus actinomycetemcomitans* (Aa), P54649; *Bacillus subtilis*, NP391861; *Bacteroides fragilis (Bact)*, P58476; *Escherichia coli* (*Ecoli*), NP286214.

	S6803	*Anabaena*	*S7942*	*Thermo*	*Aa*	*Bacillus*	*Bact*	*Ecoli*
S6803	_	68	63	64	22	22	34	23
Anabaena		_	67	69	22	23	33	23
S7942			_	63	23	20	36	23
Thermo				_	25	22	37	25
Aa					_	34	22	76
Bacillus						_	24	35
Bact							_	23
Ecoli								_

Table 3. *E. coli* and *Synechocystis* strains and plasmids used in this study.

Strains	Genotype and/or description	Source or reference
JM109		Promega
JMhtpG	JM 109 cell containing plasmid PGEMT-htpG	This study
JMhtpG::cat	JM 109 cell containing plasmid PhtpG::cat	This study
JMhsp16.6::Kan	JM 109 cell containing plasmid Phsp16.6::Kan	Laboratory stock
ΔhtpG	*Synechocystis htpG* null mutation	This study
Δhsp16.6	*Synechocystis hsp16.6* null mutation	This study
ΔhtpG::hsp16.6	*Synechocystis hsp16.6* and *htpG* double null mutation	This study
Plasmids		
pPZP111	Cm^r, for cloning *cat* gene cassette source	[3]
PGEMT-*htpG*	Amp^r, pGEMT vector containing *htpG* fragment	This study
pHTPG-cat	Amp^r and Cm^r, *htpG* was mutated	This study
pHSP16.6-kan	Amp^r and Kan^r, *hsp16.6* was mutated	Laboratory stock