HtpG is essential for the thermal stress management in cyanobacteria

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Abstract The heat shock protein (Hsp) HtpG is a member of the Hsp90 protein family. We cloned a single-copy gene encoding a homologue of HtpG from the unicellular cyanobacterium *Synechococcus* sp. PCC 7942. Sequence alignment with HtpGs from other prokaryotes revealed unique features in the cyanobacterial HtpG primary sequence. A monocistronic mRNA of the *htpG* gene increased transiently in response to heat shock. In order to elucidate the role of HtpG in vivo, we inactivated the *htpG* gene by targeted mutagenesis. Although the mutation did not affect the photoautotrophic growth at 30 and 42°C, the mutant cells were unable to grow at 45°C. They lost both basal and acquired thermotolerances. These results indicate that HtpG plays an essential role for the thermal stress management in cyanobacteria, the first such an example for either a photosynthetic or a prokaryotic organism.

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Key words: Cyanobacterium; Heat shock protein; Molecular chaperone; Hsp90; HtpG; Thermotolerance

1. Introduction

Hsp90 proteins constitute one of the most conserved heat shock protein (Hsp) families [1–3]. Hsp90 or HtpG, which is the term for a prokaryotic homologue of the Hsp90 protein, is present in the cytoplasm of eubacteria, yeast and higher organisms. Homologues also exist in the endoplasmic reticulum [1–3]. As in the cases of other molecular chaperones, Hsp90 recognizes non-native proteins and prevents irreversible aggregation during refolding [4], and thermal unfolding [5]. Hsp90 can maintain a non-native substrate in a soluble 'folding-competent' state which, upon addition of either Hsp70, Hsp40 and ATP/ADP or GroEL, GroES and ATP, leads to refolding [6,7]. In vivo, eukaryotic Hsp90 appears to be a dedicated chaperone for only a few target proteins, such as steroid hormone receptors and protein kinases [1–3].

Relatively little is known about Hsp90 homologues in prokaryotes. There are only a few studies on prokaryotic HtpG, most of which have been performed with the *Escherichia coli* HtpG. The *E. coli* HtpG has physicochemical and functional properties which are similar to its eukaryotic homologues [5,8,9]. However, its in vivo functions, although not clear, may be quite different from those of its eukaryotic counterparts. HtpG is not essential for *E. coli* because a deletion or insertion mutation is not lethal under both non-stress and thermal stress conditions [10]. Similarly, mutational studies showed that HtpG is dispensable for thermal stress management in *Actinobacillus actinomycetemcomitans* and *Bacillus subtilis* [11,12]. These results are in contrast to those with *Saccharomyces cerevisiae*, where mutations that disrupt genes encoding two nearly identical Hsp90 homologues are lethal regardless of the growth temperature [13].

Cyanobacteria are oxygenic photosynthetic prokaryotes that are phylogenetically related to chloroplasts of photosynthetic eukaryotes. They have photosynthetic electron transport systems which are functionally and structurally similar to those in chloroplasts of higher plants. Cyanobacteria, like other organisms, synthesize a diverse range of Hsps upon exposure to high temperatures [14–18]. However, functions of few of these proteins have been identified unambiguously and so far, there has been no study on cyanobacterial HtpG. As a preliminary to understand the function of HtpG in cyanobacteria, we cloned the *htpG* gene from the unicellular cyanobacterium *Synechococcus* sp. PCC 7942 and inactivated the *htpG* gene by targeted mutagenesis. The mutation in the cyanobacterial *htpG* gene has no effect on growth at normal temperatures but causes cell death even at moderately high temperatures, indicating an indispensable role of the HtpG for survival under heat stress. This is the first report of targeted inactivation of *htpG* or Hsp90 from a photosynthetic organism.

2. Materials and methods

2.1. Organisms and culture conditions

Prof. Susan S. Golden of the Texas A & M University kindly provided a wild-type strain of *Synechococcus* sp. PCC 7942. The cells were cultured photoautotrophically in BG-11 inorganic liquid medium [19] or on BG-11 plates containing 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate. The BG-11 was modified to contain 5 mM Tes/NaOH, pH 8.0, and 50 mg/l Na_2CO_3. The liquid culture in a flat glass vessel was continuously aerated. Unless otherwise indicated, cultures were grown at 30°C with a light intensity of 30 μmol/m^2/s.

2.2. DNA sequencing

Single-stranded DNA, which was generated with M13KO7 as a helper phage, was sequenced using an AutoRead Sequencing kit (Pharmacia, Uppsala, Sweden) and a DNA sequencer (DSQ-1, Shimadzu, Kyoto, Japan). The nucleotide sequences were aligned and analyzed using GENETYX software (Software Development, Tokyo, Japan).

2.3. Probe preparation and genomic library screening

A portion of the *htpG* gene from *Synechococcus* sp. PCC 7924 was cloned during the amplification of a small Hsp gene by a polymerase chain reaction (PCR). One of the PCR primers, 5′-GG(AG)C-CITT(CT)(CA)GIGA(AG)GA-3′, was based on the amino-terminal sequence of HspA of *Synechococcus vulcanus* [18], EPFREED, and the other primer, 5′-ATIC(GT)IA(GA)IGTIA(GA)IACICC-3′, was based on the internal amino acid sequence of Hsp18 of *Streptomyces albus* [20], GVLTLRI. Two major products of 0.3 and 0.4 kbp were amplified from the *Synechococcus* sp. PCC 7942 genome (600 ng) after 90 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C and extension for 2 min at 72°C.

The 0.4 kbp DNA fragment was labelled with [α-^{32}P]dCTP by a

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Abbreviations: Hsp, heat shock protein; PCR, polymerase chain reaction; OD, optical density; ORF, open reading frame

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multiprime labelling method as directed by the manufacturer (Amersham Life Science), purified through a NICK Column (Pharmacia) and then used as a specific probe for screening a *Synechococcus* sp. PCC 7942 genomic library constructed in bacteriophage λ-DASH vector (a kind gift by Dr Yasunori Nakamura of the National Institute of Agrobiological Resources). Bacteriophage DNA from positive plaques was prepared by the liquid culture method and further screening was performed by Southern blot analysis [21] after digestion of the DNA by *Eco*RI. A 3.6 kbp fragment, hybridized with the above probe, was subcloned into pBluescript II KS (+) (Stratagene, La Jolla, CA, USA). Overlapping deletions of the fragment were obtained by the Erase-a-Base System (Promega, Madison, USA) and both strands of the resulting DNAs were sequenced as described above. The *Eco*RI fragment contained an open reading frame (ORF) encoding HtpG lacking the carboxy-terminal region. In order to isolate the remaining portion of the ORF, Southern blot analysis after *Bgl*II digestion of the previously obtained positive bacteriophage DNA was performed. A 3.0 kbp DNA fragment hybridized with the ^{32}P-labelled *Bgl*II-*Eco*RI fragment (P1 in Fig. 2) was subcloned and deletions of the fragment were obtained as described above. The sequences of the overlapping regions of the *Eco*RI fragment and the *Bgl*II fragment were identical, confirming that the two fragments compose the same chromosomal region.

2.4. Preparation of total RNAs and Northern blot analysis

Total RNA from *Synechococcus* sp. PCC 7942 was prepared as described previously [22]. 10 μg of total RNA per lane was electrophoresed on a denaturing 1.5% (w/v) agarose gel containing 6.6% (w/v) formaldehyde. Northern blotting and hybridization with the ^{32}P-labelled *Bgl*II-*Eco*RI fragment (P1 in Fig. 2) as a probe were performed as described previously [22]. After hybridization, the membrane was washed in 6×SSC (1×SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C for 60 min. The size of mRNA was determined using a RNA ladder (Gibco BRL, Eggenstein, Germany). The hybridization signals were detected and quantified with a BAS 1000 Mac bio-imaging analyzer (Fuji Film, Tokyo, Japan).

2.5. Disruption of htpG

A mutant strain was generated by transforming the wild-type strain of *Synechococcus* sp. PCC 7942 with DNA of a clone in which *htpG* was interrupted by a gene conferring resistance to streptomycin and spectinomycin (see Fig. 2 and also Section 3). The construct was introduced into *Synechococcus* sp. PCC 7942 by the method described previously [23]. The transformants resistant to streptomycin and spectinomycin were segregated for a few generations by a single colony selection of BG-11 plates supplemented with spectinomycin (20 μg/ml) and streptomycin (20 μg/ml).

2.6. Thermotolerance experiments

The *Synechococcus* sp. PCC 7942 cells were grown in liquid BG-11 medium to a late log phase at 30°C under a light intensity of 40 μE/m^2/s as described above and then diluted to an absorbance of 0.08 at 730 nm. For basal thermotolerance assays (Fig. 5A), 50 ml of the diluted culture was incubated at 50°C for 20 min. An aliquot of the culture before or after the high temperature treatment was serially diluted in fresh, sterile BG-11 medium. A 5 μl aliquot from each dilution was then spotted onto a BG-11 plate and the culture was grown at 30°C under a light intensity of 30 μE/m^2/s for 12 days. Acquired thermotolerance assays (Fig. 5B) were performed as above

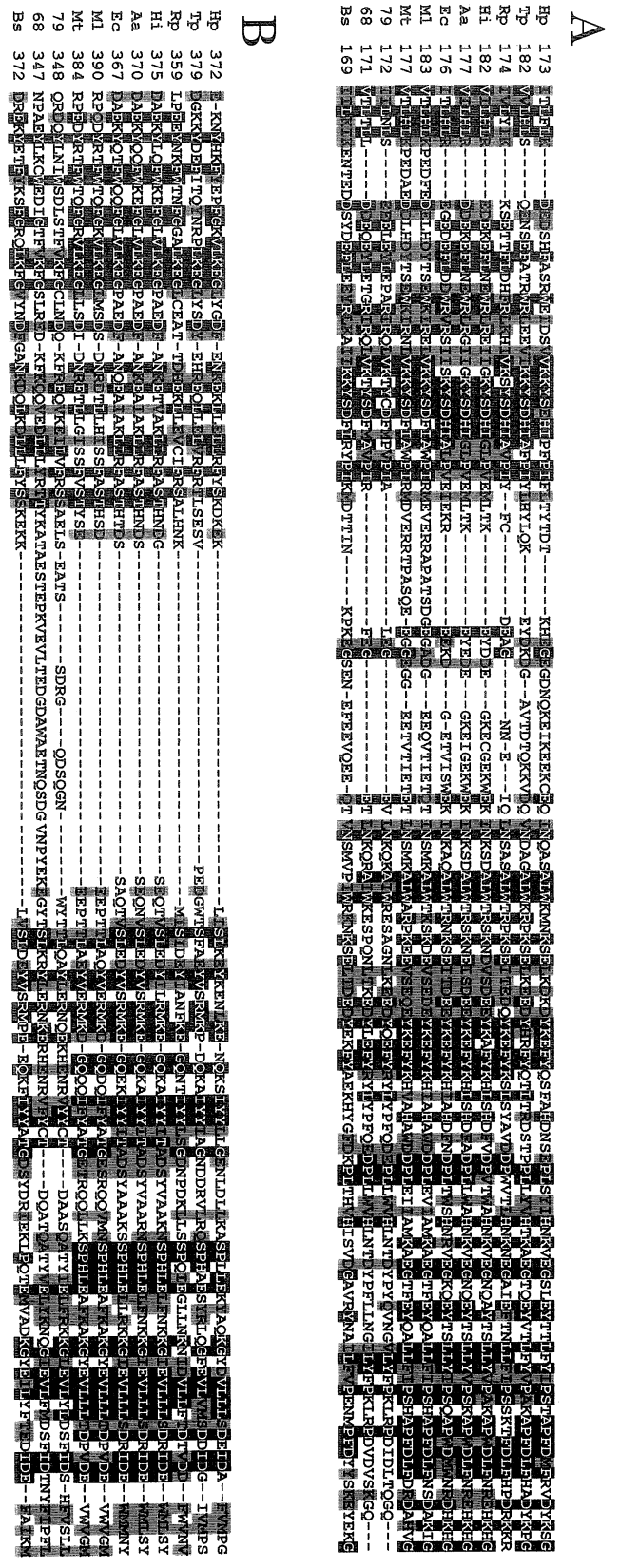

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Fig. 1. Comparison of amino acid sequences of HtpGs from *Synechococcus* sp. PCC 7942 (designated 79) and *Synechocystis* sp. PCC 6803 (designated 68) with HtpGs from other prokaryotes. Two regions, which appear to be unique only for the cyanobacterial HtpGs, are shown in A and B. The sequence of HtpG from *Synechocystis* sp. PCC 6803 was obtained through the database Cyanobase (Sll0430). The amino acid sequences from the following organisms correspond to the EMBL/GenBank/DDBJ accession numbers which are shown in parentheses immediately after the abbreviations of the organism's names: *Helicobacter pylori* (Hp: AE000511), *Treponema pallidum* (Tp: AE001265), *Rickettsia prowazekii* (Rp: AJ235273), *Haemophilus influenzae* (Hi: U32695), *A. actinomycetemcomitans* (Aa: U26968), *E. coli* (Ec: AE000153), *Mycobacterium leprae* (Ml: Z97369), *Mycobacterium tuberculosis* (Mt: Z77163), *B. subtilis* (Bs: Z99124). The sequences are aligned and represented using Clustal W [32]. Amino acid residues identical in 60 or more percent of the sequences are highlighted in black, related amino acids are gray shadowed.

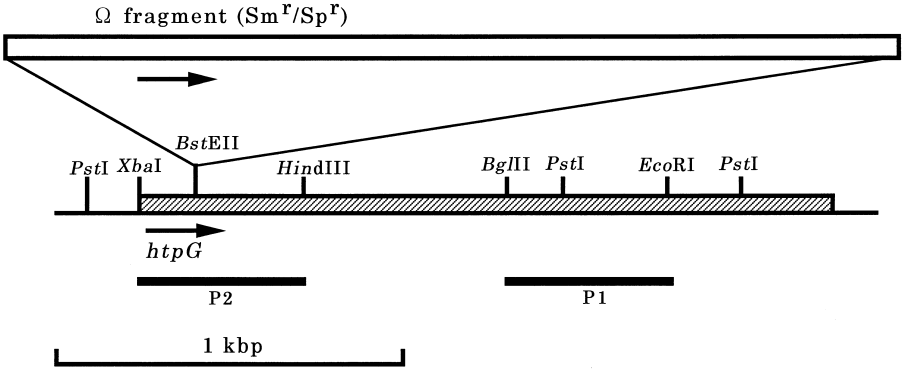

Fig. 2. Restriction map of the genomic region containing the *htpG* gene in *Synechococcus* sp. PCC 7942 and construction of a clone for insertional inactivation of *htpG*. The ORF encoding HtpG is shown by a shaded box. A spectinomycim (Sp) and streptomycin (Sm) resistant (r) gene cassette (omega fragment) which was inserted into the *Bst*EII site to inactivate the *HtpG* gene is shown by an open box. The regions of *Bgl*II-*Eco*R1 and *Xba*I-*Hin*dIII fragments, which were used for the preparation of the probes for Southern and Northern blot analyses, are indicated by bold bars, P1 and P2, respectively.

except that 50 ml of the diluted culture was given a pre-treatment at 42°C for 60 min prior to the high temperature treatment at 50°C for 20 min. In both assays, the cultures were continuously bubbled with air and the photon irradiance was maintained at 40 μE/m^2/s throughout the heat treatments at 42 and 50°C.

2.7. Nucleotide sequence accession number

The nucleotide sequence reported here has been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number AB010001.

3. Results and discussion

3.1. Cloning and sequencing of the htpG gene from Synechococcus sp. PCC 7942

Initially, we intended to amplify a small Hsp gene from the unicellular cyanobacterium *Synechococcus* sp. PCC 7942 by PCR with degenerate primers to small Hsps from other prokaryotes. Although there was no product after 30 cycles of polymerase reactions, further extended PCR resulted in a 0.4 kbp DNA fragment. One of the amino acid sequences deduced from the nucleotide sequence of the PCR product exhibited similarity to proteins belonging to the family of Hsp90 when analyzed with the National Center for Biotechnology Information BLAST network server (data not shown).

A 1914 bp ORF encoding a Hsp90 homologue of 638 ami-

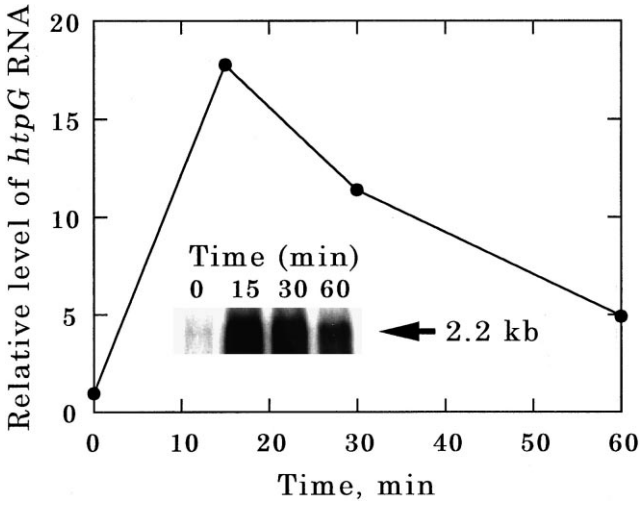

Fig. 3. Transcription of the *htpG* gene under heat stress. The transcript level was determined by Northern blot analysis of total RNA (10 μg as nucleic acid) obtained from cells incubated for different time intervals (0, 15, 30 or 60 min) after shifting cultures from 30 to 45°C. During the incubation, the liquid culture was illuminated at 30 μE/m^2/s and air-bubbled as described in Section 2.

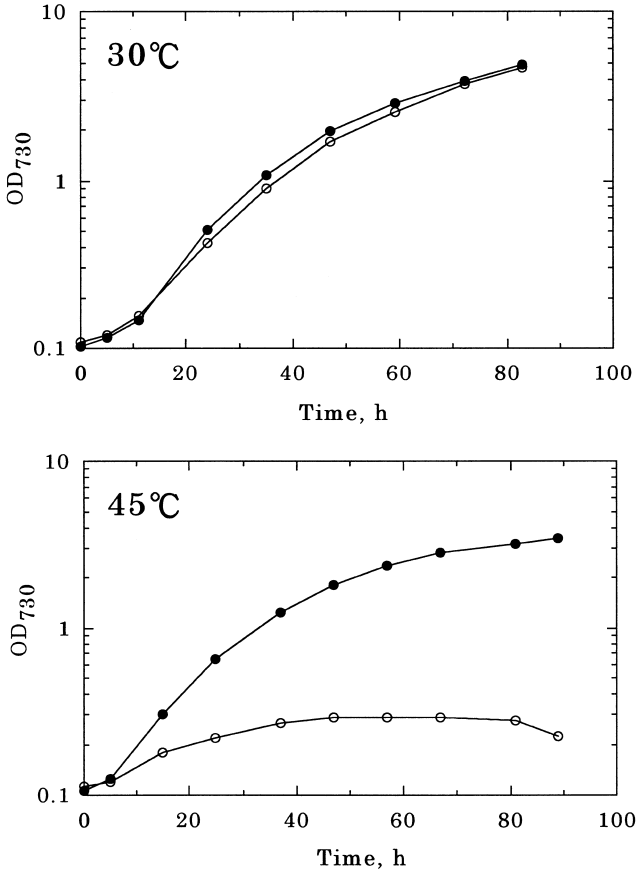

Fig. 4. Photoautotrophic growth of the wild-type (closed circles) and the mutant strain (open circles) in BG-11 liquid medium. The liquid culture was continuously aerated and illuminated at 30 μE/m^2/s. The growth of the cells at either 30 or 45°C was monitored by measuring the OD_{730} of the cultures.

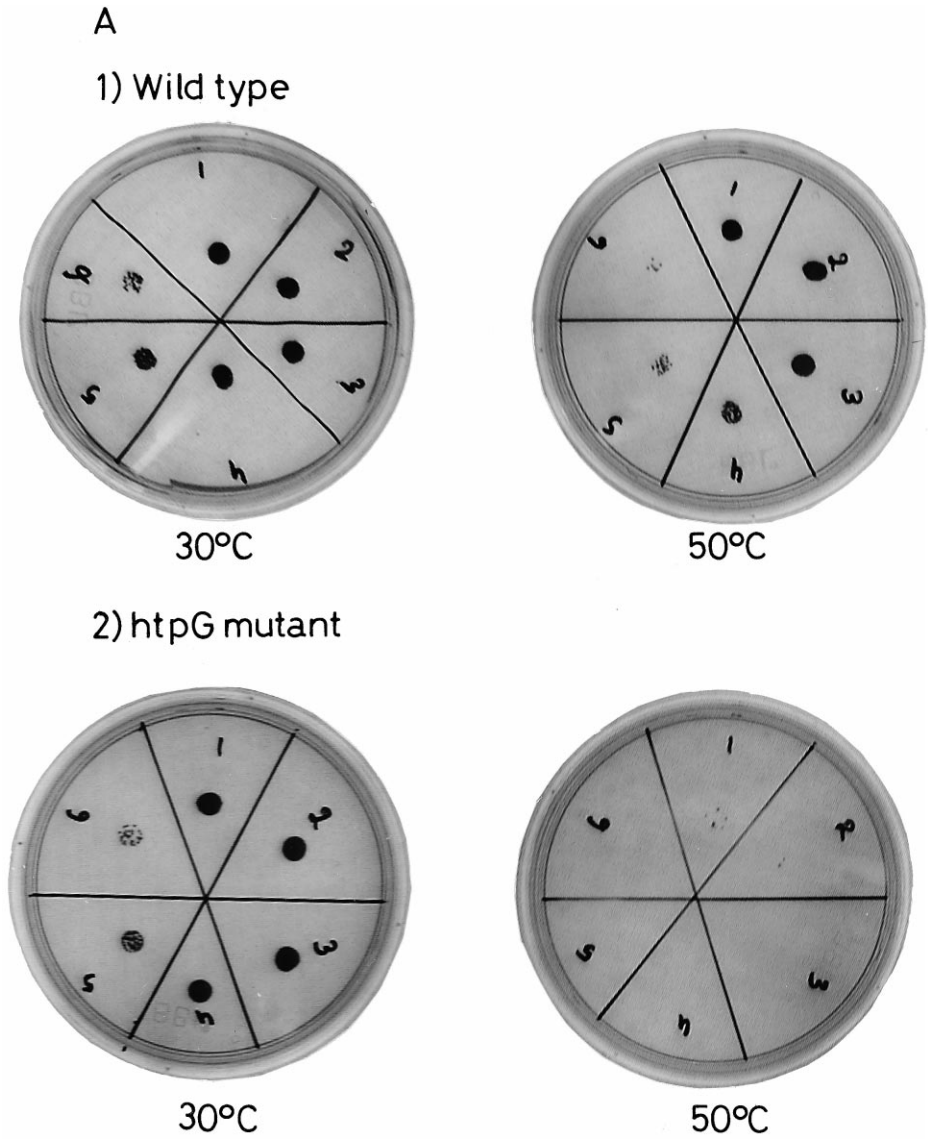

Fig. 5. The basal (A) and acquired (B) thermotolerances of the wild-type and the mutant strains. The wild-type or the mutant cells grown at 30°C (plates at the left in A) were either shifted directly to 50°C for 20 min (plates at the right in A) or first pre-incubated at 42°C for 1 hour (plates at the left in B) before being exposed to 50°C for 20 min (plates at the right in B). Cells before or after the incubation at 50°C for 20 min were taken, serially diluted (1:1, 1:5, 1:25, 1:125, 1:625, 1:3125) and spotted to each section (from 1 to 6) on a BG-11 plate.

no acids was identified in DNAs which were cloned using the PCR product as a probe to screen a *Synechococcus* sp. PCC 7942 genomic library. The PCR product was located in the region starting from amino acid number 178 of the ORF. The molecular weight and pI value for the polypeptide are predicted to be 72 602 and 4.8, respectively. These values are similar to 71 429 and 4.95 for the predicted molecular weight and pI value of the *E. coli* HtpG [8]. We designated this ORF *htpG*. An ORF, sll0430, whose deduced amino acid sequence showed the closest sequence homology (64% overall identity) among the compared sequences in the database to HtpG from *Synechococcus* sp. PCC 7942 was identified in the recently sequenced genome of *Synechocystis* sp. PCC 6803 [24].

HtpG from the two cyanobacteria exhibited less than 30% overall identity with proteins from prokaryotes and eukaryotes belonging to the Hsp90 family (data not shown). However, the amino-terminal domain of approximately 200 amino acid residues which contains the geldanamycin/nucleotide binding site [25,26] appears to be conserved in HtpGs from *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803. Four glycine residues whose mutation has been shown to diminish geldanamycin binding [27] were completely conserved in the cyanobacterial HtpGs. Comparison of 11 prokaryotic HtpG sequences including the cyanobacterial HtpGs shows that two major deletions have occurred in all the *htpG*s relative to their homologues in eukaryotic cytosol. All the prokaryotic HtpGs lack the carboxy-terminus of ~35 residues and a highly charged region which connects the geldanamycin/nucleotide binding domain with the remaining portion of Hsp90 [2,3]. Furthermore, the sequence comparison revealed two features which are unique to cyanobacterial HtpGs. One of them is that a region between position numbers 210 and 224 (numbered according to *E. coli* HtpG) did not exist in the cyanobacterial homologues (Fig. 1A). Another unique feature is that 14 and 33 amino acid residues were inserted between position numbers 409 and 410 (numbered

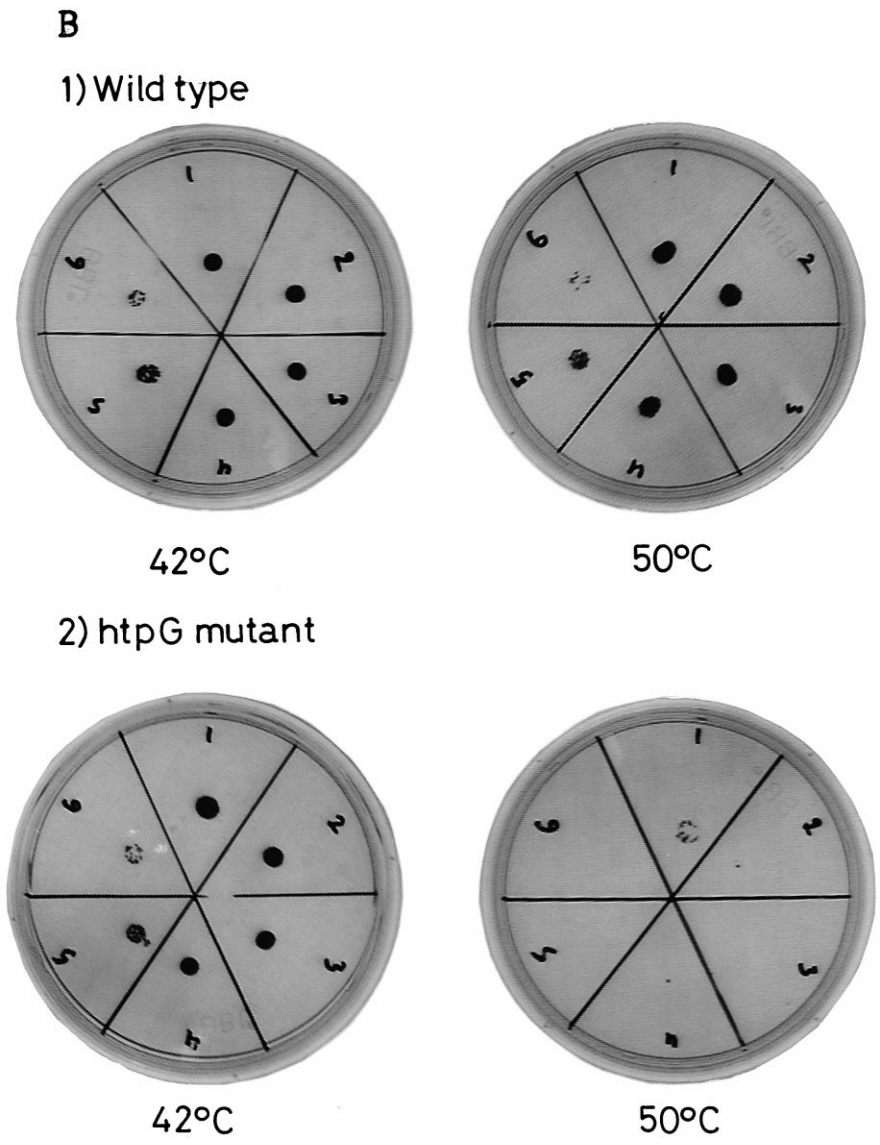

Fig. 5 (*continued*).

as above) only to homologues from *Synechococcus* sp. PCC 7942 and *Synechocystis* sp PCC 6803, respectively (Fig. 1B). It is intriguing to think that these structural features may be related to the unique function of cyanobacterial HtpGs.

3.2. Southern and Northern blot analyses

The number of *htpG* copies in the *Synechococcus* sp. PCC 7942 genome was determined by Southern blot analysis which was performed as described previously [22]. The radiolabelled 465 bp *Bgl*II-*Eco*RI fragment (P1 in Fig. 2) hybridized with only one DNA fragment when the *Synechococcus* sp. PCC 7942 genomic DNA was completely digested with *Hin*dIII, *Sal*I, *Hin*cII or *Xho*I, while the probe hybridized with two DNA fragments when the DNA was digested with *Pst*I (data not shown). There is a *Pst*I site between the *Bgl*II and *Eco*RI sites as shown in Fig. 2. These results demonstrate that there is no other gene in the genome that is extensively homologous to *htpG*.

In order to ascertain that the cyanobacterial homologue is heat-induced as eukaryotic homologues are, Northern blot analysis was carried out with the labelled *Bgl*II-*Eco*RI fragment as the specific probe. The probe hybridized with the major 2.2 kb mRNA which increased about 20-fold following a 15 min exposure to 45°C, demonstrating that *htpG* in *Synechococcus* sp. PCC 7942 is indeed a heat shock gene (Fig. 3).

3.3. Disruption of htpG

To determine whether HtpG is essential for cell growth, a cyanobacterial strain with an interruption of *htpG* was constructed and characterized. By inserting an omega fragment into the *Bst*EII site located 178 bp downstream of the initiation codon (Fig. 2), we disrupted the *htpG* gene in *Synechococcus* sp. PCC 7942. The omega fragment, which contains a streptomycin and spectinomycin resistant gene, was isolated by digesting pHP45 [28] by *Eco*RI. Two kinds of constructs were generated as judged by restriction analysis. In one construct, the omega fragment was inserted in the forward direction (the same gene orientation as that of *htpG*) and in the other construct, the fragment was inserted in the reverse direction. Both constructs were used to transform cells of nat-

urally competent *Synechococcus* sp. PCC 7942 through homologous recombination [23]. One transformant in which the omega fragment was inserted in the forward direction was selected for further analysis.

Southern blot analysis of genomic DNA from the wild-type and the mutant strain were carried out with the radiolabelled 460 bp *Xba*I-*Hin*dIII fragment as a probe (P2 in Fig. 2). When the genomic DNA was digested with *Pst*I, the probe hybridized with a 1.5 kbp fragment in the wild-type DNA, while it hybridized with a 3.5 kbp fragment in the mutant DNA (data not shown). When digested with *Pvu*II, the probe hybridized with a 3.0 kbp fragment in the wild-type DNA, while it hybridized with a 5.0 kbp fragment in the mutant DNA (data not shown). No hybridization signals corresponding to the wild-type restriction fragments were detected in the transformant. The size of the restricted DNA fragments hybridized with the probe was as expected when the omega fragment (2.0 kbp) was introduced into the *htpG* locus of all the copies of the polyploid cyanobacterial genome. The successful disruption of the *htpG* gene indicates that the *htpG* gene is not essential for normal growth in *Synechococcus* sp. PCC 7942, as previously observed for the *htpG* genes in *E. coli* [10], *A. actinomycetemcomitans* [11] and *B. subtilis* [12].

3.4. Analysis of the phenotype of the htpG mutant strain

The growth rates of the wild-type and the *htpG* mutant were measured at 30, 42 or 45°C by monitoring the optical density at 730 nm (OD_{730}). There was a little difference in growth rates at 30 and 42°C for the mutant and wild-type cultures (Fig. 4 and data not shown). However, the mutant was easily distinguished from the wild-type by the change of cell coloration from normal blue-green to yellow-green. When absorption spectra of exponentially growing cells were measured, the mutant cells showed a significant reduction in the 625 nm absorption maximum originating from phycobiliproteins as compared to the wild-type (data not shown). Thus, HtpG in photosynthetic prokaryotes may be involved either directly or indirectly in the phycobiliprotein synthesis and/or degradation. Phycobiliproteins are assembled into water-soluble, large multiprotein complexes called phycobilisomes that are located on the cytoplasmic side of the thylakoid membranes [29]. Phycobilisomes serve as the primary light-harvesting antennae for photosynthesis in cyanobacteria and red algae.

We examined whether the interruption of *htpG* may have any effect on the thermotolerance of *Synechococcus* sp. When the growth temperature was increased to 45°C, the mutant ceased to grow after a slight increase in OD_{730}, while the wild-type was able to grow under the same conditions (Fig. 4). These results indicate that the mutant cannot survive even at the moderately high temperature. In order to assess cell survival after a high temperature treatment semi-quantitatively, cells before or after the incubation at 50°C for 20 min were taken, serially diluted, spotted onto a BG-11 agar plate and cultured for 12 days. When a cell suspension before the high temperature treatment whose OD_{730} was 0.08 was 3125-fold diluted, it was possible to count the number of colonies. 37 and 30 cells were present in the diluted suspensions of the wild-type and mutant strain, respectively (Fig. 5A, 1 and 2). Therefore, approximately 100 000 cells were present initially in a 5 μl aliquot of the wild-type or mutant cell suspensions. After incubation at 50°C for 20 min, seven cells were still present in the 3125-fold-diluted suspension of the wild-type cells (Fig. 5A, 1). However, only seven cells were found in the non-diluted mutant cell suspension (Fig. 5A, 2). Thus, after the direct shift from 30 to 50°C for 20 min, approximately 20% of the wild-type cells still remained viable. In contrast, there was a dramatic loss in the mutant strain viability after the 50°C incubation, with only around 0.01% of the 30°C population remaining. It is a well-documented phenomenon that when whole organisms or cultured cells are given a short pre-treatment at a moderately elevated temperature, their resistance to killing by extreme temperatures increases. This increased resistance is known as acquired or induced thermotolerance [1]. On the other hand, resistance without the pre-treatment as already shown above is called basal thermotolerance. We examined whether the mutant cells could show an acquired thermotolerance. We employed incubation of cultures at 42°C for 60 min for a pre-treatment suitable to develop thermotolerance in *Synechococcus* sp. We think that this temperature treatment is high enough to induce *htpG* and other Hsp genes since we detected an induction of the *htpG* mRNA in this cyanobacterium even at 40°C (data not shown). In another mesophilic cyanobacterium *Synechocystis* sp. PCC 6803, *groEL* and *dnaK* mRNAs were induced to near saturation after the incubation at 42°C for 60 min [30]. After pre-treatment at 42°C for 60 min, 23 colonies appeared from a 5 μl aliquot of 3125-fold diluted cell suspensions from both the wild-type and mutant cells (Fig. 5B, 1 and 2). Thus, after the pre-treatment, most of the wild-type and the mutant cells remained viable. When shifted to 50°C for 20 min following the pre-treatment, no wild-type cells were lost (Fig. 5B, 1). However, there was no increase in the mutant strain viability even after the pre-treatment (Fig. 5B, 2). The thermotolerance assays showed that after the pre-treatment, the wild-type cells were capable of acquiring thermotolerance to some extent while the mutant cells were not.

These results with *Synechococcus* sp. are in contrast to those with *E. coli*, *A. actinomycetemcomitans* and *B. subtilis*. The *E. coli htpG* mutant was viable as described in Section 1 but had a slight growth disadvantage that increased with an increasing temperature [10]. The deletion and insertion mutants of the *E. coli htpG* gene grew more slowly than the control strain and levelled off at a lower OD at 44 or 46°C [10,31]. Similar results were obtained with the *A. actinomycetemcomitans htpG* disruptants [11]. The final extent of growth of an *htpG* insertion mutant at 42°C was reduced to 50% of the parent strain, although the growth rate appeared to be the same. Furthermore, the *htpG* null mutation in *E. coli* did not reduce cell viability at 50°C [31]. Thus, HtpG may serve only an auxiliary, growth-enhancing role in these prokaryotic cells. Recently, Versteeg et al. [12] showed that the *B. subtilis htpG* gene is not required for survival at 53°C and a *B. subtilis* insertion mutant can develop the induced thermotolerance to the same extent as the wild-type. These results contrast with our results with *Synechococcus* sp. PCC 7942. Our present study clearly demonstrated that HtpG has an essential role for thermotolerance in a cyanobacterium.

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