CURRENT MICROBIOLOGY Vol. 37 (1998), pp. 403–407

Current Microbiology
An International Journal

A 16.6-Kilodalton Protein in the Cyanobacterium *Synechocystis* sp. PCC 6803 Plays a Role in the Heat Shock Response

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Received: 28 April 1998 / Accepted: 27 July 1998

Abstract. The low molecular weight (LMW) heat shock protein (HSP) gene *hsp16.6* was identified and cloned from the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 through comparisons of genomic sequences and conserved gene sequences of the LMW HSPs. *Hsp16.6* was isolated using PCR and cloned into the pGEMT plasmid. *Hsp16.6* showed a significant increase in transcription after heat shock at 42°C that indicated *hsp16.6* was a heat shock gene. To determine the role that *hsp16.6* plays in the heat shock response, a mutant *Synechocystis* cell line was generated. Cell growth and oxygen evolution rates of wild type and mutant cells were compared after heat shock. Results showed significantly decreased cell growth rates and a 40% reduction in oxygen evolution rates in mutants after heat shock treatments. These data indicate a protective role for *hsp16.6* in the heat shock response.

At the molecular level, heat stress has deleterious effects on cell membrane function, cell metabolism, and cytoskeletal structures, as well as on the synthesis of macromolecules such as proteins and nucleic acids. However, it is known that organisms can survive heat stress through a cellular process called the heat shock response [3]. A major aspect of this cellular adaptation is the expression of a unique set of highly conserved heat shock proteins (HSPs) [13, 15, 16]. Many of these HSPs are known to function as molecular chaperones that are involved in the processes of protein folding and refolding, subunit assembly, and membrane translocation [5, 6, 18]. Other HSPs function as ATP dependent proteases which can degrade proteins [6].

Low molecular weight (LMW) HSPs have been found primarily in plants [21]. Based on amino acid sequence similarities, LMW HSPs are placed into four multigene classes [21]. Although all are nuclear-encoded, their functional locations are different in the cell. Two of the four classes (class I and II) are primarily found in the cytoplasm. Class III proteins are found in the chloroplast and class IV proteins are located in the endomembrane. Highly conserved amino acid sequences in consensus regions I and II were identified within all four classes of LMW HSPs. These conserved sequences show a high similarity with the α-crystallin eye lens protein in vertebrates [21]. Class III LMW HSPs have a unique Met-rich sequence (consensus III) that was predicted to form an amphipathic α-helix [20]. The roles of these LMW HSPs in the heat shock response are not clearly understood. Only a few plant LMW HSP homologs have been identified in prokaryotes and their functions have not been well characterized [8–10, 14].

Cyanobacteria are oxygenic photosynthetic prokaryotes that are phylogenetically and physiologically related to chloroplasts of photosynthetic eukaryotes. Cyanobacteria can readily adapt to various environmental stresses (heat, cold, oxygen depletion, lack of nutrients, salinity, and osmotic stresses). Only a few HSPs have been identified and characterized from cyanobacteria [1, 2, 4, 6, 10, 22]. A putative LMW *hsp*, encoding a 16.6-kDa protein was identified from *Synechocystis* sp. PCC 6803 by comparing conserved sequences of plant LMW HSPs with the recently sequenced genome of *Synechocystis* [11]. Northern blot hybridizations demonstrate that *hsp16.6* is heat inducible like other heat shock genes. Significant increases in heat susceptibility were observed in growth rates and O_2 evolution rates of cells with an inactivated *hsp16.6* gene after heat shock. These results demonstrate that HSP16.6 plays an important protective role against elevated temperature.

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Materials and Methods

Organism and culture conditions. *Synechocystis* sp. PCC 6803 cultures were grown in BG-11 growth medium at 27°C under constant light (photon irradiation of 30 μmol $m^{-2}s^{-1}$). Kanamycin (10 μg ml^{-1} in liquid culture and 5 μg ml^{-1} in plates) was added to the culture medium of mutant cells. Cells in early log (chlorophyll concentration of 2 to 3 μg ml^{-1}) were used for experiments.

Cloning and sequencing of *hsp16.6*. The fragment containing the putative *hsp16.6* gene (441 bp) was amplified from *Synechocystis* genomic DNA by PCR (amplimers: 5′-TGTCTCTCATTCTTTACAAT-3′, and 5′-CATTTATTAGGAAAGCTGAAC-3′) and was cloned into pGEMT plasmid for DNA sequencing analysis. DNA sequencing was performed in both directions on three different PCR clones using the dideoxy chain termination method and T7 polymerase (Amersham, Cleveland, OH, USA). Analyses of DNA and protein sequence were conducted with the PILEUP program from the University of Wisconsin's Genetics Computer Group package.

RNA Isolation and Northern blot analysis. Cells were cultured for 3 days (cell density at $A_{730nm} = 0.5$) and treated at 42°C for 1 h under 30 μmol of photons $m^{-2}s^{-1}$ before extracting mRNA [16]. RNA isolation was conducted according to the method of Odom et al. [17]. Northern blot analysis was performed by standard procedures [19]. Two and one-half micrograms of RNA was loaded into each lane. The *hsp16.6* PCR product was used as a probe. An RNA size marker (Promega, Madison, WI, USA) was used to determine the size of the transcript. After hybridization, the washed membrane was exposed to x-ray film for 15 h.

Inactivation of *hsp16.6*. The amplimers 5′-GTCGATATGCAACAATC-TGCC-3′ and 5′-CGAGTGCGAGGGAGATGGAAC-3′ (919 bp upstream and 1.2 kb downstream of *hsp16.6*, respectively) were used to amplify a 2.56-kb fragment. Inactivation of *hsp16.6* was conducted by partial deletion of *hsp16.6* and insertion of the *kanamycin* resistant gene cassette. A 454-bp fragment was excised from *hsp16.6* using two *Eco*065I endonuclease recognition sites. The excised fragment contained 214 bp of the 3′ end region of the gene and 240 bp of the down stream region. *Eco*065I endonuclease recognition sites were generated at 5′ and 3′ ends of the 1.384-kb *kanamycin* fragment using PCR (amplimers: 5′-GCGGTGACCAACGACGGCCAGTGAATTCC-3′ and 5′-TCGGTCACCCACTTTATGCTTCCGGCTCG-3′) for ligation. *Synechocystis* cells were transformed with the inactivated gene construct by the method of Williams [23]. Homologous recombination of the inactivated *hsp16.6* into *Synechocystis* genome was confirmed by PCR amplification from the genomic DNA of mutant cells, restriction endonuclease analysis, and Northern blot analysis.

Heat shock, high light, and recovery experiments. Heat shock treatments (42 and 45°C) for Northern blot, growth-rate, and O_2 evolution analyses were conducted by shifting cells from 27°C to a water bath-shaker with the selected temperatures. Heat shock treatments were conducted in room light (30 μmol of photons $m^{-2}s^{-1}$). After heat shock treatments for growth rate determinations, cultures were transferred back to 27°C where they were grown for 5 days. One-milliliter cell cultures were used for cell density readings (730 nm). High light experiments were performed by exposing cells to 300 μmol of photons $m^{-2}s^{-1}$ at 27°C for 30 min. For recovery experiments, cells were heat shocked at 45°C for 20 min and transferred back to 27°C. Oxygen evolution rates were measured after 10, 20, 30, and 60 min.

Photosynthetic measurements. A Clark-type oxygen electrode (Hansatech, UK) placed in a water circulation chamber with a constant temperature at 27°C was used to measure the photosynthetic rates of cyanobacteria cells. All the measurements were performed under constant irradiation of 80 μmol of photons $m^{-2}s^{-1}$ from an actinic light source (Quantum Devices, USA). After heat shock at 42 and 45°C, cell samples (3 ml) were directly transferred to an oxygen electrode chamber. $NaHCO_3$ was added at a final concentration of 1 mM to prevent the depletion of CO_2 during measurements.

Results and Discussion

A putative *hsp16.6* from *Synechocystis* was identified from the genomic sequence by using consensus amino acid sequences found in other LMW HSPs. The gene is 441 base pairs in length (polypeptide of 146 amino acids, molecular weight of 16.59 kDa). Alignments of the protein sequences of the putative *hsp16.6* with plant LMW HSPs showed high sequence similarities to all four plant LMW HSP classes (Fig. 1): class I, carrot *hsp18*, 55% similarity (30% identity); class II, *Arabidopsis hsp17.6*, 57% similarity (29% identity); class III, *Arabidopsis hsp21*, 53% similarity (21% identity); class IV, soybean *hsp22*, 55% similarity (29% identity). Two consensus sequences (consensus region I and II) that are highly conserved in all four classes of plant LMW HSPs were also identified in *hsp16.6* [21]. The sequence similarities of I and II in *hsp16.6* to plant LMW HSPs were higher (up to 67% in consensus I and 70% in II) than other regions of the amino acid sequence. However, the highly conserved Met-rich consensus III region in class III (chloroplast-localized) LMW HSPs could not be identified. The gene also showed a 57% similarity (37% identity) to the development-specific protein (SP21) gene found in myxobacterium, *Stigmatella aurantiaca*. SP21 is known to be a LMW HSP based on its amino acid sequence similarity to plant LMW HSPs and is expressed at high levels when myxobacteria are under stress [8, 9]. High sequence similarity of *Synechocystis hsp16.6* to LMW HSPs suggests that this gene is a member of the LMW HSP family.

To investigate the response of *hsp16.6* to high temperature, *Synechocystis* cells were shifted from 27°C to 42°C for 1 h and Northern blot analysis was conducted. Unlike other prokaryotic *hsps* (*DnaJK* and *GroELS*) that exist as operons, *hsp16.6* most likely is transcribed as a single transcript (size about 630 nucleotides). The estimated size of the *hsp16.6* transcript from Northern blot hybridization allows approximately 189 nucleotides of untranslated region. Other genes have not been identified within 266 nucleotides upstream and 1,393 nucleotides downstream region of *hsp16.6* [11]. We have identified putative promoter regions (-10 and -35) in the region upstream of *hsp16.6* and are determining the transcriptional start site by primer extension.

Heat-shocked cells transcribed *hsp16.6* mRNA while *hsp16.6* RNA could not be detected in control cells (Fig. 2, lanes 1 and 2). This demonstrates that *Synechocystis*

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hsp16.6            M SL.....ILY NPLREMDNF. ......QQQM NQLFEEVFVP
hsp18        MSIIPSFF GSRRSNVLNP FSLDIWDPF. ......QD.. ....YPLITS
hsp22    ..ANGSLLP.FM DPPITLLADL WSDRFPDPF. ......RV.. ....LEHIPF
hsp17.6            M DLGRFPIISI LEDMLEVPE. ......DH.. ....NNEKTR
 sp21           MADL SVRRGTGSTP QRTREWDPF. ......QQMQ ELMNWDPFEL
hsp21    ..KGNQGSSVEK RPQQRLTMDV SPFGLLDPLS PMRTMRQMLD TMDRMFEDTM

hsp16.6    TD..RHGDRQ G...FNPKAE LTETEEAYVL KLELPGMDPD NLDIQAARDA
hsp18      SGTSSEFGKE TAAFANTHID WKETPQAHVF KADLPGLKKE EVKVEVEEGK
hsp22      GVDKDE...A SMAMSPARVD WKETPEGHVI MLDVPGLKRE EIKVEVEENR
hsp17.6    NNPSRVYMRD AKAMAATPAD VIEHPNAYAF VVDMPGIKGD EIKVQVENDN
 sp21      ANHPWFANRQ GPPAFVPAFE VRETKEAYIF KADLPGVDEK DIEVTLTGDR
hsp21      PVSGRNRGGS GVSEIRAPWD IKEEEHEIKM RFDMPGLSKE .DVKISVEDN
                                           * **
                                                    Consensus II

hsp16.6    V.TVSGDRQD THSTEKD.GV RRTEFRYGSF RRVIPVPGAI QNTEVKANYD
hsp18      VLQISGERNK E.KEEKNNKW HRVEFSSGKF LRRFRLPENA NVDEVKAGME
hsp22      VLRVSGERKK E.EEKKGDHW HRVERSYGKF WRQFRLPQNV DLDSVKAKLE
hsp17.6    VLVVSGERQR ENKENEGVKY VRMERRMGKF MRKFQLPENA DLDKISAVCH
 sp21      V.SVSGKRER EKREESE.RF YAYERTFGSF SRAFTLPEGV DGDNVRADLK
hsp21      VLVIKGEQKK EDSDDS.... .WSGRSVSSY GTRLQLPDNC EKDKIKAELK
           *  *** *   *                          *      * * *
                                                    Consensus I

hsp16.6    AGILTLTLPK VEEAKNKVVK VQLS*..
hsp18      NGVLTVTVPK V...EMKKPE VKSIHIS
hsp22      NGVLTLTLDK LSPGKIKGPR VVSIAGE
hsp17.6    DGVLKVTVQK LPPPEPKKPK TIQVQVA
 sp21      NGVLTLTLPK RPEVQPKRIQ VASSGTE
hsp21      NGVLFITIPK .TKVERKVID VQIQ...
            **** *  *
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Fig. 1. The alignment of amino acid sequences of *hsp16.6* and LMW HSPs. HSP16.6: *Synechocystis*; HSP18: carrot, Class I (cytoplasmic); HSP22: soybean, Class IV (endoplasmic); HSP17.6: *Arabidopsis*, Class II (cytoplasmic); SP21: Myxobacterium; HSP21: *Arabidopsis*, Class III (chloroplast). Consensus sequence regions I and II are underlined and identical amino acids are indicated by an asterisk (*).

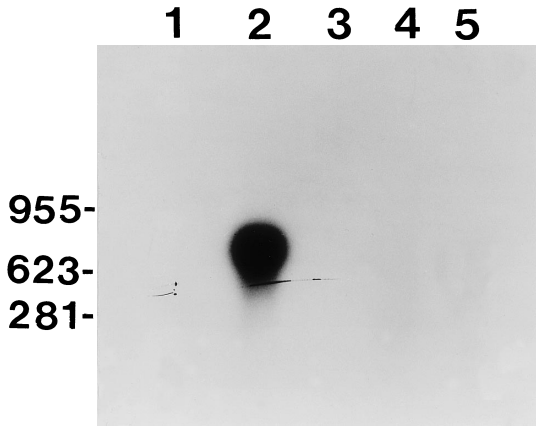

Fig. 2. Northern blot hybridization and the level of RNA in wild type and mutant cells. Lanes 1 and 2, wild type cells at 27°C and after heat shock at 42°C for 1 h; lanes 3 and 4, mutant cells at 27°C and after heat shock at 42°C for 1 h; lane 5, wild type cells after exposure to 300 µmol photons $m^{-1}s^{-1}$ for 30 min. RNA was quantified and 2.5 µg was loaded into each lane. Size markers are in nucleotides.

hsp16.6 is a heat shock gene. Since it is thought that HSP16.6 is associated with thylakoids during heat stress (10) and high light and heat are often present together under natural conditions, we hypothesized that *hsp16.6* expression may be induced by both heat and light. Cells exposed to 300 µmol photons $m^{-1}s^{-1}$ (10 times normal laboratory light for culture incubation) at 27°C transcribed a small amount of *hsp16.6* mRNA compared to heat shocked cells (Fig. 2, lanes 2 and 5). Higher light may have a greater effect on the transcriptional level.

Inactivation of *hsp16.6* in cells was demonstrated by Northern blot analysis (Fig. 2, lanes 3 and 4). No *hsp16.6* RNA was detected in mutant cells before and after heat shock. *Hsp16.6* could not be PCR-amplified from the mutant genome and the appropriate mutant construct was confirmed by PCR (data not shown).

A significant difference in growth rates was observed between mutant and wild type cells after heat shock (Fig. 3A). A 1 h heat shock at 45°C decreased the growth rate of mutant cells for at least 5 days after heat shock.

Photosystem II (PS II) is very sensitive to heat damage [6, 7, 20]; therefore, oxygen evolution is a good physiological indicator. Oxygen evolution rates were measured from wild type and mutant cells after various lengths of exposures to 42 and 45°C. After heat shock, mutant cells showed a greater reduction in oxygen evolution rates than wild type cells (Figs. 3B and C). After 20 min at 42°C, the rate of oxygen evolution in

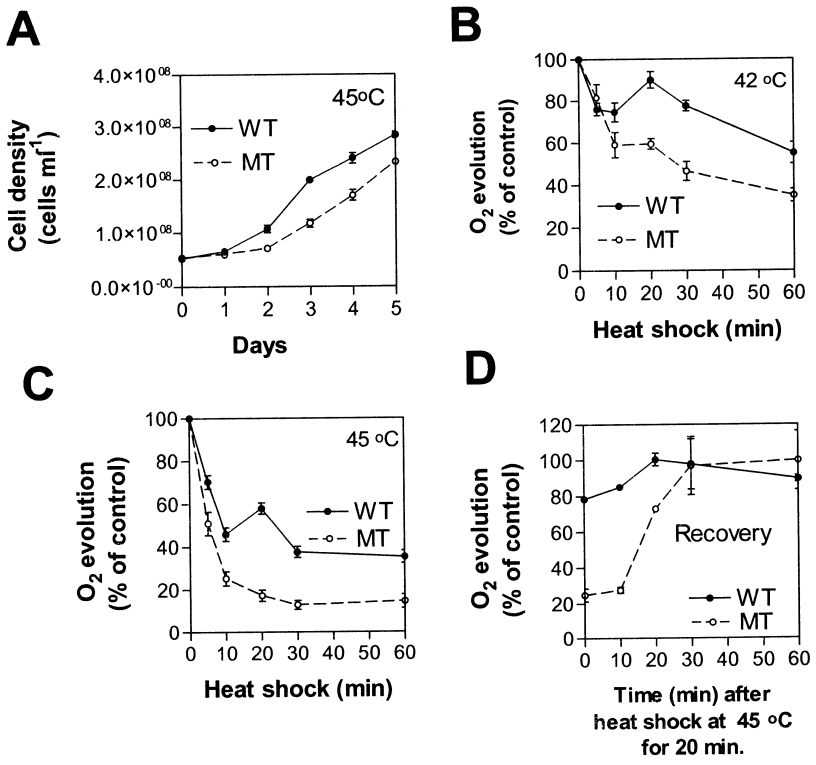

Fig. 3. Cell growth rates (A), oxygen evolution rates (B and C), and oxygen evolution rates during cellular recovery (D) in wild type and mutant cells after heat shock. Growth rates of wild type and mutants were compared for 5 days after a 1 h heat shock (A). Average values ± standard deviations from two different experiments were plotted. Oxygen evolution rates were measured after heat shock (B and C). Average values ± standard deviations from three different experiments were plotted as a percentage of the control values. Oxygen evolution rates during cellular recovery after heat shock for 20 min at 45°C (D). Average values ± standard deviations from two different experiments were plotted. WT, wild type; MT, mutant.

mutant cells was 1.18 ± 0.004 μmol of O_2 mg^{-1} of chlorophyll min^{-1}, a 40% reduction in the oxygen evolution rate before heat shock (1.97 ± 0.09 μmol of O_2 mg^{-1} of chlorophyll min^{-1}) (Fig. 3B). However, after the same treatment in wild type cells, oxygen evolution was 90% (1.96 ± 0.08 μmol of O_2 mg^{-1} of chlorophyll min^{-1}) of the nonheat shock rate (2.17 ± 0.04 μmol of O_2mg^{-1} of chlorophyll min^{-1}). After a 20 min heat shock at 45°C, oxygen evolution rates decreased even further. Oxygen evolution in mutant cells was only 18% (0.46 ± 0.14 μmol of O_2 mg^{-1} of chlorophyll min^{-1}) of the original rate (2.56 ± 0.07 μmol of O_2 mg^{-1} of chlorophyll min^{-1}) and the rate in wild type cells was 60% (1.49 ± 0.12 μmol of O_2 mg^{-1} of chlorophyll min^{-1}) of the nonheat shock rate (2.48 ± 0.1 μmol of O_2 mg^{-1} of chlorophyll min^{-1}). These results clearly demonstrate that mutant cells are more heat sensitive.

Oxygen evolution rates during cellular recovery were also monitored in wild type and mutant cells. A significantly faster recovery was observed in wild type cells (Fig. 3D). These results indicate an important role for HSP16.6 in the cellular heat shock response of *Synechocystis*.

The functions of LMW HSPs are not yet understood; however, some LMW HSPs may provide thermostability to thyladoid membranes [7, 12, 21]. A recent study by Horvath et al. demonstrated that *hsp16.6* (*hsp17*) responded to a change in thylakoid membrane fluidity [10]. The study also suggested that after heat shock, most newly synthesized HSP16.6s are associated with thylakoid membranes. Therefore, it is highly likely that HSP16.6 binds to thylakoid membrane-associated proteins and protects the integrity of thylakoids during heat shock. Studies to elucidate the function of HSP16.6 and its relationship to thylakoid membranes and to PS II are currently being conducted.

ACKNOWLEDGMENTS

This research was partially funded by grants from Ohio Board of Regents Academic Challenge, Department of Botany, and the Graduate School of Miami University. We gratefully acknowledge Dr. Alfredo Huerta for providing the oxygen electrode.

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