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Characterization of the *dnaK* Multigene Family in the Cyanobacterium *Synechococcus* sp. Strain PCC7942

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The cyanobacterium *Synechococcus* sp. strain PCC7942 has three *dnaK* homologues (*dnaK1*, *dnaK2*, and *dnaK3*), and a gene disruption experiment was carried out for each *dnaK* gene by inserting an antibiotic resistance marker. Our findings revealed that DnaK1 was not essential for normal growth, whereas DnaK2 and DnaK3 were essential. We also examined the effect of heat shock on the levels of these three DnaK and GroEL proteins and found a varied response to heat shock, with levels depending on each protein. The DnaK2 and GroEL proteins exhibited a typical heat shock response, that is, their synthesis increased upon temperature upshift. In contrast, the synthesis of DnaK1 and DnaK3 did not respond to heat shock; in fact, the level of DnaK1 protein decreased. We also analyzed the effect of overproduction of each DnaK protein in *Escherichia coli* cells using an inducible expression system. Overproduction of DnaK1 or DnaK2 resulted in defects in cell septation and formation of cell filaments. On the other hand, overproduction of DnaK3 did not result in filamentous cells; rather a swollen and twisted cell morphology was observed. When expressed in an *E. coli dnaK756* mutant, *dnaK2* could suppress the growth deficiency at the nonpermissive temperature, while *dnaK1* and *dnaK3* could not suppress this phenotype. On the contrary, overproduction of DnaK1 or DnaK3 resulted in growth inhibition at the permissive temperature. These results suggest that different types of Hsp70 in the same cellular compartment have specific functions in the cell.

All living organisms respond to environmental stresses such as high temperature by synthesizing a set of proteins which have been called heat shock proteins (Hsps) (23). Some of them are highly conserved in the course of evolution, especially the proteins encoded by the *groEL* (*hsp60* or *cpn60*) and the *dnaK* (*hsp70*) genes. They have been identified and characterized in various organisms as well as major cellular compartments, including cytoplasm, nucleus, endoplasmic reticulum, mitochondria, and chloroplasts (11, 14, 23). Although the synthesis of Hsp70 is enhanced under various stress conditions, many Hsp70 proteins are constitutively expressed and have also been shown to be essential under normal growth conditions (11, 14). One of the well-established functions of Hsp70 is the regulation of protein-protein interactions. The folding and assembly of proteins require the action of protein factors termed molecular chaperones (7, 8), and the Hsp70 family is one of the ubiquitous groups of such factors (11, 14).

Cyanobacteria are prokaryotic cells which carry a complete set of genes for oxygenic photosynthesis similar to that found in chloroplasts of higher plants. These organisms are also interesting from the point of view of evolution, since they are associated with prehistoric ages and have survived various environmental conditions. Under natural conditions they inhabit areas with suitable amounts of sunlight, where they are inevitably subject to a variety of stresses such as UV irradiation and high temperature. Therefore, it is interesting to study the mechanism of stress response and the function of stress proteins of cyanobacteria. We have previously identified three *dnaK* homologue genes, *dnaK1, dnaK2*, and *dnaK3*, in the transformable cyanobacterium *Synechococcus* sp. strain PCC7942 (27, 28).

The genome of another cyanobacterium, *Synechocystis* sp. strain PCC6803 (17), also disclosed the presence of three *dnaK* homologues (open reading frame [ORF] designations in the database are sll0058, sll0170, and sll1932). Those three DnaK homologues show high similarity to each of the three DnaKs of *Synechococcus* sp. strain PCC7942. *Synechococcus* DnaK3 has a characteristically very long C-terminal region (28), and the corresponding *Synechocystis* DnaK (sll1932) also contains this region, including the conserved GWDDDDDD/EWF sequence at the termini. The genome of the cyanobacterium *Anabaena* sp. strain PCC7120 (http://www.kazusa.or.jp/cyano) also revealed the presence of three *dnaK* homologues, although they have less similarity to each DnaK gene of *Synechococcus* than those of *Synechocystis*. Other than the cyanobacteria, the existence of multiple *dnaK* genes is rare among prokaryotes, only two other examples have been identified so far. One is *Escherichia coli*, which also has two *dnaK* homologues (*hsc66* and *hsc62*) other than *dnaK* (20, 36, 45), and the other is *Borrelia burgdorferi* B31 (9), which has two *dnaK* homologues. In most eukaryotes, Hsp70s constitute a multigene family whose members have been shown to be expressed differentially under a variety of physiological conditions (23, 42). Some are expressed constitutively and are not induced by stress, and others are both constitutive and stress inducible. However, it is not clearly understood how these Hsp70 proteins in the same cellular compartment are assigned with their respective functions. In *Saccharomyces cerevisiae*, most of the cytosolic Hsp70 proteins belong to either the Ssa or Ssb subfamily. Those which belong to the same subfamily have com-

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pensatory functions for each other, but those from different subfamilies are not interchangeable (5, 6, 43).

Although chaperone functions are well characterized using certain substrates, it has been a major subject in recent studies to understand how molecular chaperones find their specific target among many substrates in a specific cellular process. It would contribute to this subject to clarify how the different Hsp70s in the same cellular compartment are allocated to their functions. As an initial step for studying the functional distinction among spatially colocalized Hsp70s, we analyzed in vivo functions and gene regulation of the three DnaK proteins in *Synechococcus* sp. strain PCC7942. Here we report the various properties of these proteins, which suggest that each protein has a specific function(s) in the cell.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Synechococcus* sp. strain PCC7942(R2-spc), which has been cured of its indigenous plasmid pUH24 (19), was obtained from T. Endo (Nagoya University, Nagoya, Japan). Cells were grown photoautotrophically at 30°C in BG-11 medium (4) under bubbling with air and continuous illumination. When necessary, media were supplemented with kanamycin at a final concentration of 10 μg/ml. *E. coli* MC4100 (3) and NRK156 (18) cells were grown in Luria-Bertani (LB) medium at the indicated temperatures. Media were supplemented with ampicillin at a final concentration of 100 μg/ml if required.

Disruption of *dnaK* genes. Plasmid pDK1A, which has 2,698-bp DNA fragment including the *dnaK1* gene (27), was used for gene disruption of *dnaK1*. The kanamycin resistance gene was isolated as a *Bam*HI fragment from pUC4K (Pharmacia) and inserted into the *Hin*cII site within the *dnaK1* gene of pDK1A to make pDK1KM. Similarly, plasmid pDK2KM, which has the kanamycin resistance gene inserted between the *Bst*XII and *Bgl*II sites within the *dnaK2* gene, was constructed. To avoid single-cross recombination, pDK1KM and pDK2KM were linearized by digestion with *Eco*RI, and the linearized product was used to transform *Synechococcus* sp. strain PCC7942 according to the method described by Porter et al. (31). For *dnaK3* gene disruption, three DNA fragments upstream and downstream of *dnaK3* and promoterless kanamycin resistance cassette were amplified by PCR using primers which have 5′ add-on sequences designed to create overlapping sequences among those fragments and were recombined by a recombinant PCR technique (15), as shown in Fig. 1. The resulting fragment frDK3KM was transformed into *Synechococcus*. Transformants were grown on BG-11 agar plate containing kanamycin (10 μg/ml).

Protein purification and preparation of antisera. DNA fragments encoding the C-terminal domain of each DnaK protein (amino acids [aa] 520 to 655 of DnaK1, aa 515 to 634 of DnaK2, and aa 516 to 685 of DnaK3) were amplified by PCR. Those fragments of *dnaK1* and *dnaK2* were inserted in the *Eco*RI and *Sal*I sites of the hexahistidine fusion expression vector pET21b (Novagen). Similarly, the fragment of *dnaK3* was inserted in the *Bam*HI and *Hin*dIII sites of the hexahistidine fusion expression vector pQE10 (Qiagen). C-terminal segment of each DnaK protein was purified using Ni-nitrilotriacetic acid (NTA) affinity resin (Qiagen) according to the manufacturer's instructions. Purified proteins were injected into mice. The mice were boosted after 3 weeks and bled for serum preparation 4 weeks later.

Western blotting analysis. Crude extracts of *Synechococcus* sp. strain PCC7942 cells were prepared as follows. Nine-milliliter aliquots of cell culture were mixed with 1 ml of 100% trichloroacetic acid (TCA). After incubation for 15 min or more on ice, precipitates were harvested by centrifugation at 6,000 × *g* for 10 min. To remove TCA, the pellet was washed successively with 500 μl of 100% acetone and then 500 μl of 100% ether and then solubilized with 90 μl of 10 mM Tris-HCl (pH 7.5)–2% sodium dodecyl sulfate (SDS)–20 mM NaOH. The debris was removed by centrifugation at 10,000 × *g* for 1 min, and the supernatant was used as the crude extract. Part of the crude extract was measured for protein concentration and one-fourth volume of 5× modified sample buffer (250 mM Tris-HCl [pH 6.8], 500 mM dithiothreitol, 0.5% bromophenol blue, 2% SDS, and 50% glycerol) for SDS-polyacrylamide gel electrophoresis (PAGE) was added to the extract and boiled for 3 min. Protein concentration was determined by the Lowry method with bovine serum albumin as the standard. The proteins were separated by SDS-PAGE (12.5% polyacrylamide) and transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore). For the detection of DnaK proteins, mouse antiserum against each DnaK protein was used as the primary antibody, and horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG; heavy and light chains) antibody (sheep; Amersham) was used as the secondary antibody. Horseradish peroxidase activity was detected by color development using a substrate kit (Bio-Rad). To detect GroEL protein, rabbit antiserum against *Bacillus subtilis* GroEL (H. Yoshikawa, unpublished) was used as the primary antibody, and alkaline phosphatase-conjugated anti-rabbit IgG antibody (goat; Biomarker) was used as the secondary antibody. Color development associated with alkaline phosphatase was performed using nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as described (33).

Pulse-labeling experiments. *Synechococcus* sp. strain PCC7942 cells were grown in BG-11 at 30°C, and at the logarithmic growth phase (optical density at 700 nm [OD_{700}] of 0.5), the culture temperature was shifted to 45°C. At appropriate intervals, 10-ml aliquots of the culture were sampled and pulse labeled with 100 μCi of [^{35}S]methionine (Amersham) at the culture temperature for 30 min. At the end of the labeling, 1.1 ml of 100% TCA (final, 10%) was added and incubated on ice for 15 min. Precipitates were then harvested by centrifugation at 6,000 × *g* for 10 min, washed with acetone and ether, and dissolved in 200 μl of 50 mM Tris-HCl (pH 7.5)–2% SDS–20 mM NaOH. The solution was mixed with 1 ml of 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–2% Triton X-100–1 mM EDTA, and the debris was removed by centrifugation at 16,000 × *g* for 5 min. Supernatant was divided in four, and immunoprecipitation was carried out with four kinds of antisera. To each aliquot, 750 μl of 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–2% Triton X-100–1 mM EDTA and 4 μl of each antiserum were added, and the mixture was incubated overnight at 4°C. To each sample, 50 μl of 10% (wt/vol) IgGsorb was added, and the mixture was kept at 4°C for 20 min. After centrifugation at 10,000 × *g* for 1 min, the pellets were successively washed with 0.5 ml of 50 mM Tris-HCl (pH 7.5)–1 M NaCl–1% Triton X-100 and with 0.5 ml of 50 mM Tris-HCl–0.5 M NaCl–0.05% SDS. The washed pellets were resuspended in 20 μl of 1× sample buffer for SDS-PAGE (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 0.1% bromophenol blue, 2% SDS, 10% glycerol) and then boiled for 3 min. To seperate proteins from the IgGsorb, the mixture was centrifuged at 10,000 × *g* for 1 min, and the supernatant was loaded on SDS-PAGE (12.5% polyacrylamide). Bands were detected using Bio-Imaging analyser BAS2000 (Fujix).

Oligonucleotides and construction of plasmids. Oligodeoxyribonucleotides were synthesized using an ABI392 DNA/RNA synthesizer (Perkin Elmer Applied Biosystems Japan). Sequences of the oligonuculeotides used for construction of *dnaK* overexpression plasmids are as follows: TRC1-N, GCGAGCTCT AAGGAGGTAATTTATGGGCAAGGTTATC; TRC1-Na, GCGAATTCTAA GGAAAAAATTTATGGGCAAGGTTATC; TRC1-C, GCTCTAGACTACTC AATCGCCTCGTAGTC; TRC2-N, GCGAGCTCTAAGGAACTGGACTAT GGCCAAAGTTGTC; TRC2-C, GCTCTAGATTACTTCGACTCAGAGAAC TCTGC; TRC3-N, GCGAGCTCTAAGGAGGTGACAGCATGGGACGAGT CGTAG; TRC3-Na, GCGAGCTCTAAGGAAAGACAGCATGGGACGACG AGTCGTAG; and TRC3-C, GCTCTAGACAGCCTGATCCGCCGACTGAG.

The expression vector pTrc99A/X, which carries the *lacI*^{q} gene and *trc* promoter, was obtained from T. Endo (Nagoya University) and used to express each *dnaK* gene under the control of isopropyl-β-D-thiogalactopyranoside (IPTG). This vector contains a ribosome-binding site (RBS) and an initiation codon upstream of multicloning sites. Therefore, we synthesized forward primers (TRC-N series: TRC1-N, TRC2-N, and TRC3-N for *dnaK1, dnaK2*, and *dnaK3*, respectively) which contain an in-frame termination codon (TAA) and RBS downstream of the *Sac*I site to produce intact DnaK proteins. Reverse primers (TRC-C series: TRC1-C, TRC2-C, and TRC3-C for *dnaK1, dnaK2*, and *dnaK3*, respectively) were designed to contain an *Xba*I site after the termination codon of each *dnaK* gene. A DNA fragment harboring each *dnaK* gene was amplified by PCR with the above primers, digested with *SacI* and *XbaI*, and inserted into the *Sac*I and *Xba*I sites of pTrc99A/X.

Expression of *dnaK3* in *Synechococcus* at the neutral site of the chromosome. Plasmid pNS1 is a derivative of pTZ18R containing the spectinomycin resistance cassette of pHP45 (32) in the middle of the *Synechococcus* fragment designated the neutral site (22), which allows homologous recombination between the transforming plasmid DNA and the recipient cyanobacterial chromosome to take place. A fragment containing *lacI*^{q}, *trc* promoter, and *dnaK3* was isolated from pTrcDK3 and recloned into pNS1 between the separated neutral site segments, next to the spectinomycin resistance cassette. This plasmid was used to transform *Synechococcus* sp. strain PCC7942, and spectinomycin-resistant transformants were selected on BG-11 medium containing spectinomycin (40 μg/ml). Chromosomal DNA was extracted from one of the transformants, and recombination was confirmed by PCR. This strain, which expresses *dnaK3*, was designated NSK3.

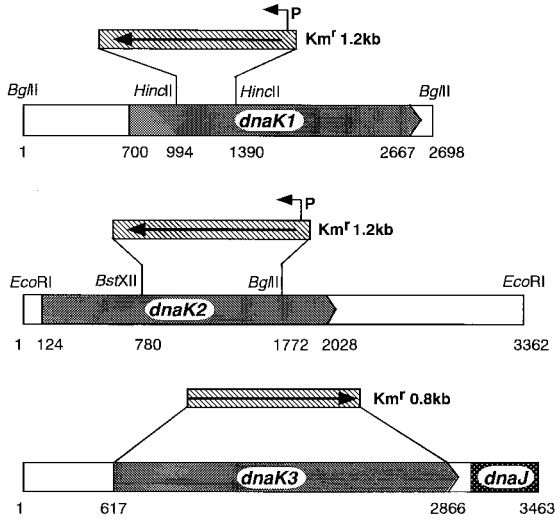

FIG. 1. Disruption of *dnaK* genes from *Synechococcus* sp. strain PCC7942 by insertion of kanamycin resistance gene. The kanamycin resistance gene (Km^r) from pUC4K was inserted into each *dnaK* gene as described in Materials and Methods. P, promoter.

RESULTS

Disruption of each *dnaK* gene. In *E. coli, dnaK* is not essential for growth, but deletion of the gene confers a temperature-sensitive (ts) phenotype (2, 30). To determine whether each *dnaK* homologue gene of *Synechococcus* sp. strain PCC7942 is essential for cell growth, we attempted to disrupt each *dnaK* gene by inserting an antibiotics resistance marker. By using plasmid pDK1KM (Fig. 1), *dnaK1* was disrupted as described in Materials and Methods. *Synechococcus* is known to contain multiple copies of the chromosome. To confirm if all the chromosomal copies of the *dnaK1* gene were changed to the mutant form, we isolated chromosomal DNA of transformants, and the *dnaK1* gene region was examined by PCR using primers TRC1-N and TRC1-C. When chromosomal DNA from the wild-type strain was used as the template, a 2.0-kb DNA fragment was detected, and a band of this size was not amplified from the DNA of transformants; instead, a 2.8-kb fragment was observed (data not shown). This increase in size corresponds to the kanamycin resistance gene insertion. Additionally, a 70-kDa protein band was no longer detected in the Western blot analysis for the crude extracts of the transformant cells using anti-DnaK1 antiserum (data not shown). These results confirmed that all chromosomal copies of the *dnaK1* genes were totally disrupted. This disruptant, named DK1KM, could grow under normal conditions. Considering that DnaK1 is a heat shock protein homologue, we examined the growth of the mutant at high temperatures and found that DK1KM could grow even at high temperatures, similar to the wild-type strain (data not shown).

In *E. coli*, mutation in *dnaK* results in high basal levels of other heat shock proteins at 30°C and failure to turn off the heat shock response at 42°C, suggesting that DnaK functions as a negative regulatory factor in the heat shock response (30, 38, 39). We therefore analyzed the effect of heat shock on the levels of DnaK2, DnaK3, and GroEL proteins in DK1KM by Western blotting. We did not detect any difference in the amount of either DnaK2, DnaK3, or GroEL protein between the wild-type and *dnaK1* mutant cells, both at basal levels and after heat shock (data not shown). Therefore, *Synechococcus* DnaK1 does not seem to function as a regulator in the heat shock response, in contrast to the *E. coli* DnaK.

We also attempted to disrupt *dnaK2* and *dnaK3* by inserting a kanamycin resistance marker. Considering that *dnaK3* may be cotranscribed with downstream $dnaJ^{7942}$, frDK3KM, which has an insertion of a promoterless and terminatorless kanamycin resistance casette, was used for the *dnaK3* gene disruption (Fig. 1). However, we could not disrupt all copies of either *dnaK2* or *dnaK3* gene in the cell. Most transformants formed extremely small colonies, and when their *dnaK2* and *dnaK3* loci were examined by PCR using primers TRC2-N and TRC2-C or TRC3-N and TRC3-C, fragments of two sizes were amplified as a mixture in each case. The size of one band corresponds to the wild-type allele, and the size of the other band represents the allele with the kanamycin resistance cassette insertion (data not shown). These results suggest that DnaK2 and DnaK3 are both essential for growth under normal conditions.

To confirm the essentiality of *dnaK3*, the same Km^r insertion-carrying *dnaK3* fragment was used to transform the NSK3 strain, which carries an IPTG-inducible intact *dnaK3* at the neutral site. Km^r transformants appeared as normal-size colonies, and all of the original *dnaK3* alleles in these cells were replaced by ones with the Km^r insertion. In these experiments, the transformants were spread onto BG-11 plates containing 0, 0.01, 0.1, and 1.0 mM IPTG to express *dnaK3* at various levels. Most of the transformants were viable without IPTG, and the number and size of the colonies decreased with increasing IPTG concentration. These findings indicate that leaky expression from the *trc* promoter produced a sufficient amount of DnaK3 protein and that a comparative increase in DnaK3 expression had a deleterious effect on the cell.

Differential accumulation and synthesis of DnaK and GroEL proteins after heat shock. To analyze the changes in the level of each DnaK protein after heat shock, we made specific antisera for each DnaK protein using less-conserved C-terminal regions. We expressed the C-terminal polypeptides of the DnaK proteins with a hexahistidine tag at their C terminus (DnaK1 and DnaK2) or N terminus (DnaK3), purified the polypeptides using Ni-NTA affinity resin, and prepared antisera against them as described in Materials and Methods. We first performed Western blot analyses for crude extracts of *Synechococcus* sp. strain PCC7942 cells and confirmed that three *dnaK* genes were actually expressed and that each antiserum was specific to each DnaK protein (Fig. 2). Then we examined the effect of heat shock on the levels of DnaK as well as GroEL protein. Cells were grown at 30°C and shifted to 45°C at the logarithmic growth phase. Crude extracts of the cells at various times were prepared and subjected to Western blot analysis. The levels of DnaK and GroEL proteins in response to heat shock varied (Fig. 3). DnaK2 protein level increased until 30 min after heat shock, and thereafter, the increased level was maintained over the period examined. The GroEL protein level also, but more markedly, increased from 0 to 100 min. Although the levels did not decrease to those before heat shock within 120 min, these two proteins exhibited a typical heat shock response. On the other hand, the level of DnaK3 protein was not affected by heat shock. Characteristically, the amount of DnaK1 protein decreased after heat

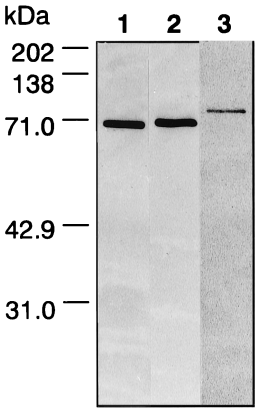

FIG. 2. Specificity of antiserum raised against DnaK proteins of *Synechococcus* sp. strain PCC7942. C-terminal regions of three DnaK proteins were purified, and antisera against these polypeptides were prepared as described in Materials and Methods. The specificity of each antiserum was tested by Western blotting of crude extracts (30 μg of protein) from *Synechococcus* cells. Lane 1, anti-DnaK1; lane 2, anti-DnaK2; lane3, anti-DnaK3.

shock. These results indicate that expression of the *dnaK* and *groEL* genes is differentially regulated upon temperature upshift. Since the band intensities of Western blots using purified proteins were almost identical (data not shown), the antibody titers of anti-DnaK antiserum seem to be comparable. Therefore, the weak intensity of the DnaK3 band reflects a relatively small amount of the protein in the cell, compared with DnaK1 and DnaK2.

In additional experiments, we measured the synthesis rate of DnaK and GroEL proteins by 30-min pulse labeling at various times after temperature upshift. The synthesis rate of DnaK1 protein was not obviously changed (Fig. 4). The results of the Western blot exhibiting a reduction in protein accumulation (Fig. 3) show that DnaK1 seems to be degraded more rapidly during heat shock. DnaK2 and GroEL proteins showed a transient but significant increase of synthesis rate after heat shock, which correlates with the result from the Western analysis. While DnaK3 protein did not show a distinct change in synthesis rate (Fig. 4), its accumulation seems to be kept constant (Fig. 3), suggesting the existence of a mechanism to maintain the level of this protein.

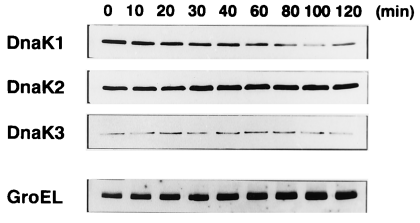

FIG. 3. Western blot analysis of DnaK and GroEL proteins after heat shock. *Synechococcus* cells were grown at 30°C and shifted to 45°C at the logarithmic phase (OD_{700} of 0.5). Crude extracts were prepared from cultures at the indicated times after temperature upshift, and samples (30 μg of protein for DnaK and 10 μg of protein for GroEL) were analyzed by Western blotting using antiserum specific to each DnaK protein or anti-*B. subtilis* GroEL antiserum as described in Materials and Methods.

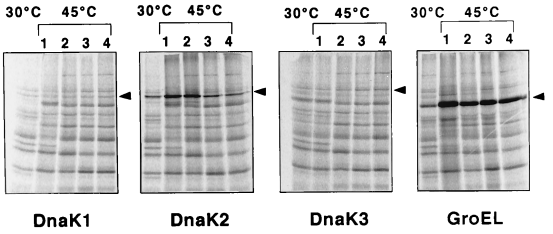

FIG. 4. De novo synthesis of DnaK and GroEL proteins after heat shock. Logarithmically growing *Synechococcus* cultures (OD_{700} of 0.5) were shifted from 30 to 45°C. Aliquots of the cultures were sampled before (control, first lane in each panel) or after temperature upshift at appropriate intervals (lane 1, 0 min; 2, 30 min; 3, 60 min; and 4, 90 min after temperature upshift) and pulse labeled with [^{35}S]methionine at the indicated temperatures for 30 min. Proteins were immunoprecipitated with either DnaK or GroEL antiserum and separated by SDS-PAGE. Bands were detected using Bio-Imaging analyzer BAS2000.

Heat shock response in Δ*rpoD* mutant. To discern whether each *dnaK* or *groEL* gene is transcribed by RNA polymerase holoenzyme containing any one of four principal sigma factors (RpoD1, RpoD2, RpoD3, and RpoD4), we carried out Western blot analyses on each *rpoD* mutant. Crude extracts were prepared from wild-type *Synechococcus* sp. strain PCC7942 cells and from mutant strains D1KM (Δ*rpoD1*), D2KM (Δ*rpoD2*), D3KM (Δ*rpoD3*), and D4KM (Δ*rpoD4*) (12) both before and after heat shock at 45°C for 60 min. For the non-heat-shocked samples, there was less difference in the amounts of the DnaK and GroEL proteins between wild-type cells and *rpoD* mutant cells (Fig. 5).

The responses of these proteins to heat shock in the *rpoD* mutants exhibited the same pattern as those of the wild-type cells; the amount of DnaK1 protein decreased, that of DnaK2 and GroEL increased, and that of DnaK3 was constant. RpoD2, RpoD3, and RpoD4 are not responsible for the transcription of these Hsp genes, since these *rpoD* genes are completely inactivated in strains D2KM, D3KM, and D4KM, respectively. On the other hand, RpoD1 is an essential sigma factor, and strain D1KM, in which *rpoD1* is partially deleted at its N terminus, has residual RpoD1 activity (Masuda et al., unpublished results). Therefore, the possibility that RpoD1 controls the transcription of these Hsp genes cannot be ruled out.

Differential effects of *Synechococcus* DnaK overproduction on *E. coli* cell morphology. To examine the effect of *Synecho-*

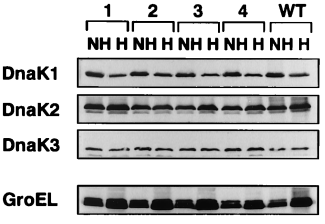

FIG. 5. Heat shock response in Δ*rpoD* mutants. Crude extracts were prepared from wild-type *Synechococcus* cells (WT) and from the *rpoD1* (lanes 1), *rpoD2* (lanes 2), *rpoD3* (lanes 3), and *rpoD4* (lanes 4) mutants, which encode each of four principal type sigma factors, both before (NH) and after (H) heat shock at 45°C for 60 min, and subjected to Western blotting. Note that RpoD1 is an essential sigma factor, and the Δ*rpoD1* mutant, in which the *rpoD1* gene is partially deleted at its N terminus, has residual RpoD1 activity.

TABLE 1. 5′ oligonucleotide sequences of primers used to construct each *dnaK* expression plasmid[a]

Plasmid	5′ oligonucleotide	Sequence[a]	Expression[c]
pTrcDK1a	DK1TRC-Na	**TAAGGA**AAAA**AT**TT (ATG)	±
pTrcDK1	DK1TRC-N	**TAAGGAGGT**A**AT**TT (ATG)	+++
pTrcDK2	DK2TRC-N	**TAAGGA**AC**TG**GA**C**T (ATG)	+++
pTrcDK3a	DK3TRC-Na	**TAAGGA**AAGACAGC (ATG)	+
pTrcDK3	DK3TRC-N	**TAAGGAGGTGA**CAGC (ATG) TAAGGAGGTGATC[b]	+++

[a] Nucleotides that are identical to the complementary sequence of the 3′ end of *E. coli* 16S rRNA are highlighted in boldface.
[b] Complementary sequence of the 3′ end of *E. coli* 16S rRNA.
[c] Symbols indicate the expression levels of each DnaK protein in the range from detectable (±) to the maximum (+++) which we obtained in Fig. 6.

coccus sp. strain PCC7942 DnaK production on cell physiology in *E. coli* and to determine whether the *E. coli dnaK756* ts phenotype can be complemented by the *Synechococcus dnaK* genes, we constructed IPTG-inducible expression plasmids for each *dnaK* in which *Synechococcus* sequences are preceded by Shine-Dalgarno sequences of *E. coli*. The sequences of the junction regions are shown in Table 1. Our initial constructs are those containing sequences TRC1-Na, TRC2-N, and TRC3-Na. Among these, cells harboring a plasmid carrying TRC1-Na and TRC3-Na produced only detectable amounts of DnaK protein (Table 1), whereas pTrcDK2 could produce distinct amounts of DnaK2 (Fig. 6). We therefore reconstructed plasmids with primers in which RBSs and the preceding sequences are modified close to the complementary sequence of the 3′ end of 16SRNA of *E. coli* (Table 1). Newly constructed pTrcDK1 and pTrcDK3 indeed drove overexpression of DnaK proteins, as shown in Fig. 6.

By using the expression plasmids described above, we examined the effect of DnaK overproduction on the cell morphology of *E. coli* MC4100. Strains expressing any of the *Synechocccccus* DnaK proteins from these plasmids were found to produce colonies of equal size to those of the control strain harboring no plasmid. We also observed that cells grown on LB plates without IPTG were indistinguishable from those containing no plasmid (Fig. 7). The addition of 1 mM IPTG, which results in overproduction of DnaK proteins (Fig. 6), led cells harboring each *dnaK* producer to exhibit aberrant morphology. A substantial population of cells producing DnaK1 or DnaK2 became filamentous (Fig. 7). Overproduction of *E. coli* DnaK has been shown to result in cell filamentation (1). From the viewpoint that overproduction leads to a defect in cell septation, DnaK1 and DnaK2 seem to exhibit a function similar to that of *E. coli* DnaK.

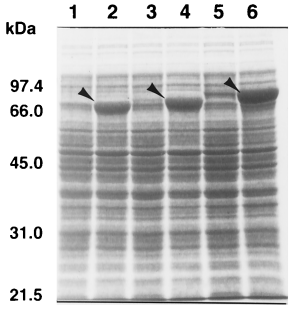

FIG. 6. Overproduction of DnaK1, DnaK2, and DnaK3 proteins in *E. coli*. *E. coli* MC4100 cells harboring the *dnaK* expression plasmids pTrcDK1 (lanes 1 and 2), pTrcDK2 (lanes 3 and 4), and pTrcDK3 (lanes 5 and 6) were grown at 37°C in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 1 mM IPTG. After incubation for 5 h, cultures were sampled and cells were harvested by centrifugation. Cells were suspended in SDS sample buffer and subjected to SDS-PAGE on 10% polyacrylamide gels, followed by Coomassie blue staining. The positions of molecular size standards are indicated on the left. Arrows indicate the positions of the DnaK proteins.

Moreover, the toxic effect of DnaK2 protein is more prominent than that of DnaK1, since extremely filamentous cells were observed just 18 h after the addition of IPTG. These two proteins seem to be produced at similar levels (Fig. 6), and therefore, the difference in extent of the toxic effect suggests a functional difference between these proteins. When DnaK3 was overproduced, filamentous cells were not seen; rather, somewhat unusual morphology was observed (Fig. 7). Cells were relatively swollen and sometimes twisted. The appearance of the colonies on an LB plate was also unique, with heterologous regions at their edges. It is possible that the plasmid was instantaneously rearranged and cells no longer overproducing DnaK3 began to grow.

Complementation of *E. coli dnaK* ts mutant. To determine whether the three DnaK proteins are functional homologues of the *E. coli* DnaK, complementation experiments were carried out using the *E. coli* NRK156 strain, which has the *dnaK756*(Ts) mutation (Table 2). NRK156 cells harboring each *dnaK* expression plasmid were grown in LB medium at 37°C, and at the logarithmic growth phase (OD_{660} of 0.2), 5×10^3 cells were spread on LB agar plates containing various concentrations of IPTG as indicated. Cells were incubated at the permissive temperature (37 or 42°C) or nonpermissive temperature (44.5°C), and colony-forming ability was determined. As summarized in Table 2, DnaK2 could suppress ts growth at 44.5°C, while DnaK1 and DnaK3 could not suppress growth at 44.5°C; rather, overproduction of these proteins resulted in growth inhibition even at the permissive temperature (42°C). This inhibitory effect was not seen in the absence of IPTG and became more severe as the IPTG concentration was increased. Therefore, the effect is likely the consequence of overproduction of DnaK1 or DnaK3. DnaK3 seems to be more toxic, since overproduction of DnaK3 inhibited growth at relatively lower IPTG concentrations than DnaK1. The growth inhibition was not observed at 37°C even in the presence of 1 mM IPTG, indicating that this effect is temperature dependent.

DISCUSSION

We could not completely disrupt all copies of either *dnaK2* or *dnaK3* in *Synechococcus* cells, as our results indicate. This strongly suggests that these two genes are essential for normal

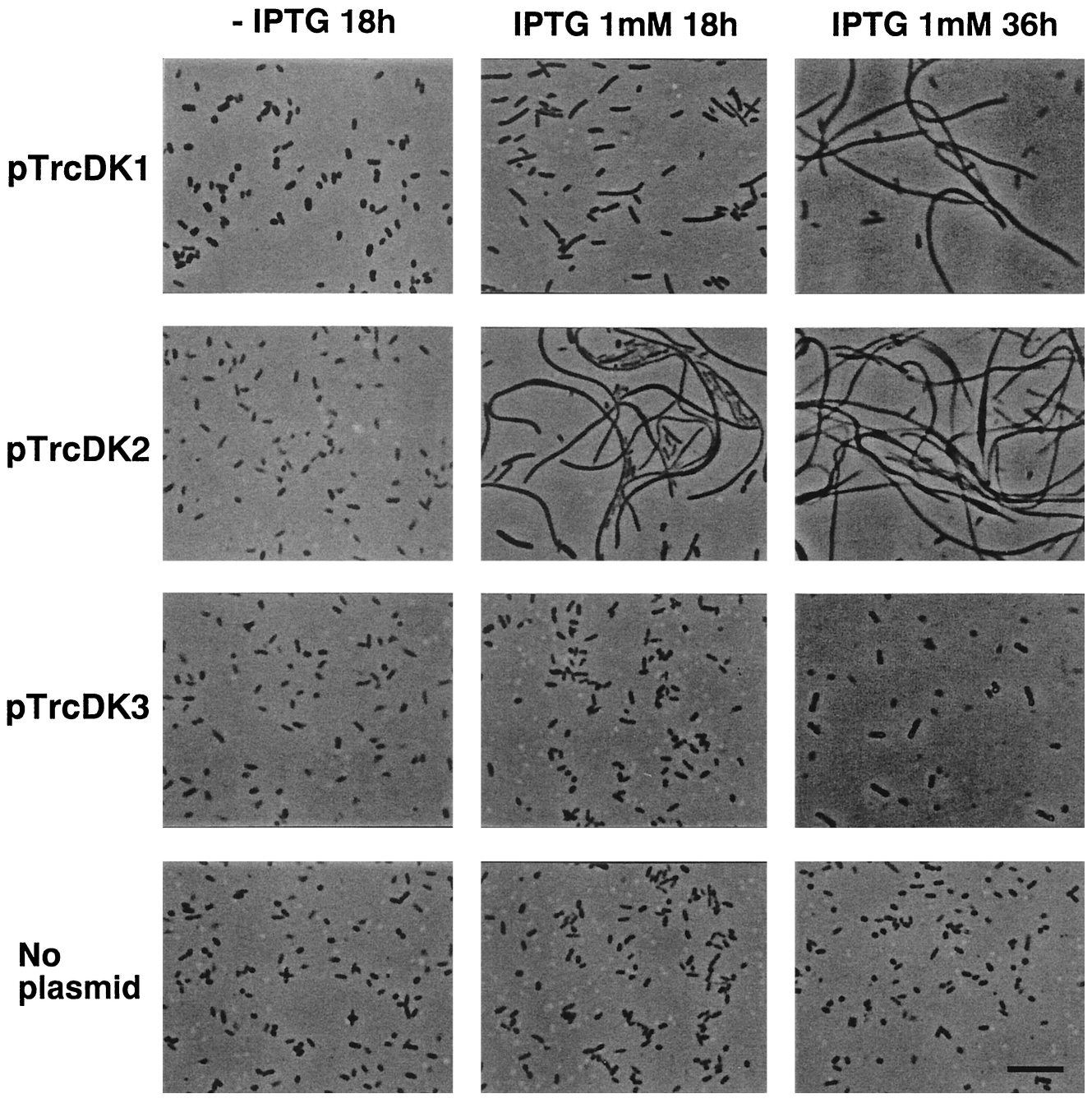

FIG. 7. Effects of *Synechococcus* DnaK overproduction on *E. coli* cell morphology. *E. coli* MC4100 cells harboring *Synechococcus dnaK* expression plasmids were grown on LB agar plates in the presence or absence of 1 mM IPTG. After incubation for 18 and 36 h at 37°C, cells from multiple representative colonies were examined under a microscope (BX60; Olympus) with a U Plan F1 objective lens (×40), and photographs were taken with Neopan 400 Presto films (Fujifilm). In the absence of IPTG, the cells showed no difference in morphology after incubation for 18 and 36 h. As controls, MC4100 cells without plasmid were similarly grown on LB agar plates and examined after incubation for 18 and 36 h. Bar, 10 μm.

growth and that DnaK2 and DnaK3 have some specific function which cannot be compensated for by the remaining two DnaK proteins. There have been no previous reports mentioning that two species of Hsp70 in the same cellular compartment are both essential. In *Saccharomyces cerevisiae*, 15 genes of the Hsp70 family which encode proteins localized in different cellular compartments have been found. Among them, Ssa and Ssb represent the two classes of abundant cytosolic Hsp70s. The essential Ssa proteins are encoded by four genes, *SSA1* to *SSA4*. The Ssb proteins are encoded by the *SSB1* and *SSB2* genes. Though Ssbs are not essential for growth (6), the Ssa and Ssb families have been suggested to be functionally distinct; overexpression of an Ssa protein fails to rescue the phenotype of an *ssb* mutant, and vice versa (5). A chimera con-

TABLE 2. Effect of *Synechococcus dnaK* expression in *E. coli dnaK756*(Ts) mutant

IPTG (mM)	Growth[a]											
	37°C				42°C				44.5°C			
	No plasmid	pTrcDK1	pTrcDK2	pTrcDK3	No plasmid	pTrcDK1	pTrcDK2	pTrcDK3	No plasmid	pTrcDK1	pTrcDK2	pTrcDK3
0	+	+	+	+	+	+	+	+	−	−	−	−
0.1	NT	NT	NT	NT	NT	+	+	+	NT	−	+	−
0.5	NT	+	+	+	NT	+	+	±	NT	−	+	−
1.0	NT	+	+	+	NT	±	+	−	NT	−	+	−

[a] +, growth; −, no growth; NT, not tested.

sisting of the N-terminal ATPase domain from Ssa1 protein and the remainder from Ssb1 protein was shown to be able to rescue the phenotype of *ssb1 ssb2* cells (16). The N-terminal regions of DnaK2 and DnaK3 bearing the ATP-binding domain and the following ca. 100 amino acids are quite similar, and the remaining C-terminal regions are variable. Since DnaK3 is characteristic in that it has a relatively long C-terminal nonconserved region, it would be interesting to discern whether this C-terminal region is responsible for functional specificity.

We have shown here that each DnaK protein exhibits a characteristic accumulation pattern and synthesis rate after heat shock, suggesting that the three *dnaK* genes are differentially regulated. The different responses after heat shock suggest functional differences between Hsp70 proteins in a multigene family. In our study, the synthesis of DnaK1 was constitutive but the protein was found to be degraded after heat shock. This property, similar to that of Ssb proteins in *S. cerevisiae*, seems instead to reflect a general response of ordinary proteins after heat shock.

The level of DnaK2 increased in response to heat shock, like that of GroEL and other typical Hsp proteins. In prokaryotic cells, the regulation of heat shock response in *E. coli* has been well studied and is known to be under the control of the *rpoH* gene product, a σ^{32} transcription factor (10, 13, 38, 48). However, σ^{32} might not be a universal regulator of the heat shock response in prokaryotes, since no σ^{32}-related factor or σ^{32}-specific heat shock promoter has been found in many other bacteria (47). Instead, a novel inverted repeat termed CIRCE has been found between the transcriptional and translational start sites of heat-inducible genes in some gram-positive bacteria, including *Bacillus subtilis* and cyanobacteria (21, 44, 49). In *B. subtilis*, *groE* is transcribed by RNA polymerase holoenzyme containing the principal σ^{A} factor, and its heat-inducible transcriptional start sites are the same as those at low temperature and are preceded exclusively by vegetative promoters (21, 34, 40). From in vivo and in vitro studies, the inverted repeat (CIRCE) has been suggested to serve as an operator, and both *dnaK* and *groE* operons have been postulated to be negatively regulated by a repressor encoded by the *hrcA* gene (24, 35, 46, 49). The Western blot analyses for four principal sigma factor mutants of *Synechococcus* sp. strain PCC7942 suggested that three *dnaK* and *groEL* genes are transcribed by RNA polymerase holoenzyme containing either principal sigma factor RpoD1 or another, if any, minor sigma factor. In *Synechococcus* sp. strain PCC7942, σ^{32}-related factor has not been found, but CIRCE is conserved upstream of the *groESL* operon (41). Therefore, it is possible that the *dnaK2* gene also contains a CIRCE element in the promoter region and is transcribed by RNA polymerase holoenzyme containing the principal RpoD1 factor. However, no CIRCE element was found in the upstream region of any of the three *dnaK* genes of *Synechocystis* sp. strain PCC6803, although it is conserved in both the *groESL* operon and *groEL-2* gene. Alternatively, cyanobacterial *dnaK* genes may have another regulatory mechanism that dose not depend on CIRCE or σ^{32}.

The synthesis rate of DnaK1 and DnaK3 did not show any distinct change after temperature upshift. After heat shock, the DnaK3 level was kept constant, while DnaK1 seems to be degraded more rapidly, suggesting the existence of a mechanism to maintain the level of DnaK3 protein. Gene disruption experiments indicated that DnaK3 is essential for growth. DnaK3 therefore appears to play an important role in cell physiology under normal conditions other than heat shock response.

Although *E. coli dnaK* is dispensable under normal growth conditions, mutation in *dnaK* causes ts growth at both high and low temperatures and defects in septation, which result in cell filamentation (2, 30). *E. coli dnaK* mutants also survive poorly during carbon starvation and stationary phase (37). Overproduction of DnaK also results in the formation of filamentous cells and reduced cell viability during stationary phase (1). It is interesting to note that both DnaK deficiency and overproduction result in similar physiological defects. Overproduction of the FtsZ protein in *dnaK* mutants suppressed filamentation, suggesting that DnaK might play a role in cell division via some action on FtsZ (2). Defective septation in strains overproducing DnaK may also result from an interaction(s) between DnaK protein and proteins involved in cell division (1). Since overproduction of *Synechococcus* DnaK1 or DnaK2 in *E. coli* resulted in cell filamentation, it is possible that DnaK1 and DnaK2 interact with *E. coli* proteins involved in cell division. When expressed in the *E. coli dnaK756* mutant, *dnaK2* could suppress the growth deficiency at the nonpermissive temperature, while *dnaK1* and *dnaK3* could not. On the contrary, overproduction of DnaK1 or DnaK3 resulted in growth inhibition at the permissive temperature. Taken together, these results suggest that DnaK2 is a functional counterpart of the *E. coli* DnaK protein.

The *E. coli* genome project has disclosed two other *dnaK* homologs in this organism. However, neither of these seems to be structurally related to any *dnaK* gene of *Synechococcus*. It should be noted that both DnaK2 and DnaK3 contain several motifs specific to Hsp70 proteins in chloroplasts and significant

amounts of DnaK3 are localized to the thylakoid membrane (26). Moreover, a *dnaJ* homologue gene (*dnaJ*^{7942}) which is located immediately downstream of *dnaK3* has also been identified and characterized (29). *dnaJ*^{7942} is essential for growth, and DnaJ^{7942} is also detected quantitatively in the thylakoid membranes. These facts suggest that DnaK3 and DnaJ^{7942} cooperatively have some specific function(s) related to photosynthesis. *Synechocystis* sp. PCC6803 was revealed to have four *DnaJ* homologues (ORF designations in the database are sll0897, sll1666, sll1933, and slr0093). Among them, *dnaJ*^{6803} (sll1933) is similarly located downstream of *dnaK*^{6803} (sll1932), which corresponds to *Synechococcus dnaK3*, and DnaJ^{6803} (sll1933) shows similarity with DnaJ^{7942} in that they do not have a Gly/Phe-rich domain or a zinc finger domain, both of which are often identified in DnaJ homologues. These two genes seem to be cotranscribed and to be the only pair which constitute an operon among the three *dnaK* and four *dnaJ* genes. It is therefore intriguing to assume that DnaK3/DnaJ^{7942} is involved in localizing proteins required for photosynthesis to thylakoid membranes.

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