Three different DnaK proteins are functionally expressed in the cyanobacterium *Synechocystis* sp. PCC 6803

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Multiple dnaK genes appear to be common in cyanobacteria; the function of the encoded proteins is, however, still elusive. To characterize the dnaK gene family from the cyanobacterium *Synechocystis* sp. PCC 6803 in detail, genetic analyses were combined with analyses of the expression and localization patterns of the three encoded proteins. While significant expression of all three genes was found, the results obtained clearly indicate physiological differences of the three proteins in *vivo*, and DnaK2 seems to have a key function in *Synechocystis*. Expression of DnaK3 appears also to be as essential as expression of DnaK2, whereas the dnaK1 gene was deleted without resulting in any distorted phenotype. In line with a suggested privileged function, expression of DnaK2 altered most significantly after heat shock.

INTRODUCTION

Proteins of the 70 kDa heat-shock protein family (Hsp70) are ubiquitously distributed in all kingdoms of life, and in eukaryotes members of this family can be found in several organelles as well as in the cytosol (Bukau & Horwich, 1998; Bukau et al., 2006). The main functions of the proteins are to ensure proper folding of polypeptide chains, to keep proteins in a translocation-competent state after synthesis, and to prevent misfolding and aggregation of proteins caused by e.g. cellular stress. Although some Hsp70 proteins are involved in the cellular heat-shock response, the proteins are usually constitutively expressed and functional, and therefore members of the Hsp70 family are better described as chaperones rather than as heat-shock proteins (Rospert & Chacinska, 2006).

Besides the structure of an almost complete bovine Hsc70 protein (Jiang et al., 2005), the structure of the ~45 kDa N-terminal ATPase domain has been resolved recently (Flaherty et al., 1990; Harrison et al., 1997; Zhang & Zuiderweg, 2004) as well as the structure of the 25 kDa peptide-binding domain (Cupp-Vickery et al., 2004; Wang et al., 1998; Zhu et al., 1996), which is linked to the ATPase domain via a short flexible linker. The C-terminal part of Hsp70 proteins is not well conserved, and the physiological function of this domain is less characterized.

In recent years, the Hsp70 protein from *Escherichia coli* (DnaK) has become a paradigm for studying the activity and function of Hsp70s, and the general mechanism of DnaK activity has been mainly investigated in *E. coli*. Interestingly, the dnaK gene can be easily deleted in *E. coli*, although the deletion strain displayed defects in cell growth at low and high temperatures (Bukau & Walker, 1989a, b; Paek & Walker, 1987). The ATPase activity of *E. coli* DnaK is regulated by the two co-chaperones DnaJ and GrpE. DnaJ enhances the substrate-binding activity of Hsp70 by stimulating its ATPase activity, whereas when ADP is bound the protein binds tightly to substrates (Harrison, 2003; Liberek et al., 1991).

While in eukaryotes several different Hsp70 proteins exist in the various cellular compartments, in bacteria usually only a single DnaK protein is present. Nevertheless, in a few bacteria more than one DnaK homologous protein exists, and in *E. coli*, for example, two DnaK-homologous proteins can be found besides the canonical DnaK (Levilt & Kawula, 1995; Seaton & Vickery, 1994; Yoshimune et al., 1998). While this is rather uncommon in other bacteria, all cyanobacteria appear to encode several DnaK proteins as deduced from the available genomic sequence information. A dnaK multigene family was originally identified in the cyanobacterium *Synechococcus* sp. PCC 7942 (Nimura et al., 1994a, b, 1996, 2001), but later on it became evident that also in the genomes of other completely sequenced cyanobacteria dnaK gene families exist, the functions of which are still largely unknown. As in *Synechococcus*, in the genome of the mesophilic
cyanobacterium Synechocystis sp. PCC 6803 three dnaK genes can be found, which encode highly homologous proteins (Kaneo et al., 1996). In contrast to Synechococcus, it has recently been proposed that only one dnaK gene is functionally expressed in Synechocystis whereas the other two genes are silent (Varvasovszki et al., 2003).

Here we present an analysis of the expression and function of the three DnaK proteins in Synechocystis, and the results contrast with some observations made in Synechococcus. Since Synechocystis can grow under various experimental conditions and can be easily genetically modified, it appears to be an excellent model organism to study the functions of a cyanobacterial DnaK family. Based on immunological analysis we found that all three DnaK proteins are expressed in Synechocystis, although deletion analyses indicated that the function of DnaK2 and DnaK3 is physiologically of greater importance than the function of DnaK1. Furthermore, only for the dnaK2 gene expression did we observe a heat-stress response typical for heat-shock protein genes.

**METHODS**

**Cell culture.** A glucose-tolerant Synechocystis sp. PCC 6803 wild-type strain (kind gift of M. Rögner, Bochum, Germany) was maintained in liquid BG11 medium (Rippka et al., 1979) at 34 °C under 33 μE m⁻² s⁻¹ of fluorescent cold white light. For cultivation of mutant strains, 30 μg kanamycin ml⁻¹ was added to the medium. All cultures were grown photoautotrophically unless otherwise indicated. E. coli strain DH5α was used for plasmid propagation. Temperature-sensitive E. coli strains ΔdnaK52 and ΔdnaK756 were maintained at 30 °C.

**Preparation of cellular protein fractions.** Synechocystis cells were harvested at exponential growth phase (OD₇₅₀ 0.5–2.0). Pelleted cells were resuspended in 50 mM HEPES pH 8.0 and broken with glass beads (0.25–0.5 mm diameter) in a Mini Beadbeater. To prevent protein degradation a proteinase inhibitor mix was added to the cell suspension. Glass beads and unbroken cells were removed by centrifuging twice at 16 000 g. Soluble and membrane protein fractions were obtained by ultracentrifugation of the total cell extract at 48 000 g for 30 min. Pelleted membranes were washed with HEPES buffer and resuspended in the same volume as the supernatant.

**Alkali extraction of membrane proteins.** A Synechocystis wild-type cell culture grown in the presence of 5 mM glucose was harvested at exponential growth phase (OD₇₅₀ ≈ 1). The pellet was resuspended in 50 mM HEPES/KOH pH 8.0 and total cell extract was prepared as described above. The extract was divided into 1000 μl samples and membranes were sedimented by ultracentrifugation. The supernatant was discarded and the pelleted membranes were washed and then resuspended in 500 μl HEPES buffer (control) or in 500 μl of the same buffer containing either 100 mM NaOH or 100 mM Na₂CO₃. After incubation for 30 min on ice, membranes were recovered by ultracentrifugation. The supernatant was collected and the membranes were resuspended in the same volume of HEPES buffer. Equal volumes of soluble and corresponding membrane protein sample were subjected to SDS-PAGE and Western analysis.

**Electrophoresis and Western blotting analysis.** Proteins were separated by SDS-PAGE using 8 % polyacrylamide gels according to standard procedures (Sambrook et al., 1989). For Western analysis, proteins were transferred onto PVDF membranes. The individual DnaK proteins were detected with primary antibodies, raised in rabbits (see Fig. 1 for the peptide sequences used), and an anti-rabbit IgG alkaline-phosphatase-conjugated secondary antibody.

**Disruption of dnaK genes.** All molecular methods were done by standard procedures (Sambrook et al., 1989).

To disrupt the individual dnaK genes, the individual genomic regions were amplified by PCR using the following primers: NtdnaK1 (5'-ataagcatctggtggcagcgggatcg-3'), CtdnaK1 (5'-ttgctagattgctctggattg-3'), NtdnaK2 (5'-ataagcatctggtgctgggtttc-3'), CtdnaK2 (5'-ttgctagattgctctggattg-3'), NtdnaK3 (5'-ataagcatctggtgctgggtttc-3'), CtdnaK3 (5'-ataagcatctggtgctgggtttc-3'). The isolated fragments were ligated into the restriction-digested plasmid pSET6a (Schoepfer, 1993), and a kanamycin resistance cassette (aphA), derived from the plasmid pBSL14 (Alexeyev, 1995), was inserted into the genes via SmaI (dnaK1 and dnaK2) or HindIII (dnaK3) restriction sites. The constructs were transformed into Synechocystis wild-type cells (Williams, 1988) and positive clones were selected on BG11 agar plates containing 10 μg kanamycin ml⁻¹. Positive clones were continuously transferred onto selective agar plates containing increasing concentrations of kanamycin (up to 100 μg ml⁻¹) for more than a year. To check whether the deletion strains were completely segregated, genomic DNA was isolated and the corresponding regions were analysed by PCR and restriction analysis. For the isolation of the dnaK genomic regions the following primers were used: NtdnaK1, CtdnaK1, NtdnaK2, CtdnaK2, NtdnaK3, CtdnaK3check (5'-gtttggacagcgggaaaggtg-3') and CtdnaK3check (5'-ctctgggctgaaaccattg-3')

**Heterologous expression of Synechocystis DnaK proteins in E. coli.** The individual dnaK genes were amplified by PCR using the primers NtdnaK1, CtdnaK1HindIII (5'-ataagcatctggtgctgggtttc-3'), NtdnaK2, CtdnaK2, NtdnaK3 and CtdnaK3. The isolated fragments were ligated into a modified version of the pBAD24 expression vector (Guzman et al., 1995) via the Ndel/HindIII restriction sites (dnaK1 and dnaK3) or via Ndel/BamHI (dnaK2). For protein expression, these constructs were transformed into E. coli ΔdnaK52 (Bukau & Walker, 1989b). Cultures were grown at 30 °C and DnaK protein expression was induced at an OD₆₀₀ of 0.6 with 0.2 % arabinose. After 6 h cultures were harvested and broken by sonication. Unbroken cells were removed from the extracts by centrifugation at 1600 g.

**Generation of dnaK reporter strains.** The promoter regions of each dnaK gene were amplified by PCR and introduced upstream of the luxAB reporter genes on the promoter-probe vector pILA (Kunert et al., 2000) via the PstI/KpnI restriction sites. The constructs were transformed into Synechocystis wild-type cells, and positive clones were selected and further treated as described above. For calculation of background luminescence a control strain (luxAB without any promoter fused) was also generated.

**Luminescence measurement of dnaK promoter activity.** Measurement of dnaK promoter activities was performed using a Lumat LB 9507 luminometer (Berthold Technologies). Synechocystis cells were diluted in fresh BG11 medium to an OD₇₅₀ of 0.5. Luciferase reactions of 1 ml cell suspensions were measured after addition of 1 mM decanal (50 mM stock solution in 100 % ethanol), and luminescence was measured for 30 s.

**Complementation of an E. coli TS ΔdnaK strain.** The E. coli dnaK gene was cloned using the primers NtdnaKEC (5'-ctctagatggtggacttattcgag-3') and CtdnaKEC (5'-ggccgaagcttctggattg-3'), and genomic E. coli DNA as template. The genes encoding the E. coli DnaK protein and Synechocystis DnaK proteins (see above) were cloned into the pMal-c2 (New England Biolabs) vector. The resulting libraries were transformed into the E. coli strain MC1061ΔTS (Kunert et al., 2000).
Biolabs) expression plasmid via NdeI/HindIII restriction sites. The resulting plasmids were transformed into *E. coli* Δdnak756 (Georgopoulos *et al.*, 1979) and cells were grown at 30°C. A 50 ml culture volume of each transformant was used to inoculate cultures of 3 ml LB containing ampicillin and various concentrations of IPTG, and the cultures were subsequently incubated at either 30°C or 42°C overnight. Cell growth was followed by measuring the OD600. For the determination of the DnaK protein localization, cellular protein fractions were prepared from cultures grown at 30°C.

**Expression of a C-terminally truncated DnaK3 version.** Genes encoding the full-length DnaK3 as well as a C-terminally truncated DnaK3 version were amplified using the primers NtdnaK3SalI (5'-atatgcgtcgaccctttgtcaccagtggtttgc-3') and CtdnaK3SpeI (5'-atagcactagtattggaacctgctgcacagctgc-3') or CtdnaK3shortSpeI (5'-atatgcactagtattggaacctgctgcacagctgc-3'), respectively. The resulting fragments were ligated into the plasmid pILA (Kunert *et al.*, 2000) via the SalI/XbaI restriction sites. The construct for the complete deletion of the genomic *dnaK3* gene was generated as follows. The region 400 bp upstream of the *dnaK3* gene was amplified using the primers NtdnaK3SalI and CtdnaK3us (5'-ccatgttgttttctttgctcc-3') and ligated into the TA cloning vector pCR2.1 (Invitrogen). The 400 bp downstream region of the *dnaK3* gene was amplified using the primers NdtnaK3ds (5'-atacggttctaatgtcaaaagttcg-3') and CtdnaK3ds (5'-atacgctcgaggccattgacacttgttccg-3'). The resulting fragment was ligated into the construct harbouring the upstream *dnaK3* region via the EcoRV/XhoI restriction sites, corresponding to a direct orientation of both *dnaK3* flanking regions. A chloramphenicol resistance cassette was amplified from the plasmid pACYC184 (New England Biolabs) using the primers NtcmR (5'-cactttgcgccgaataaatacc-3') and CtcmR (5'-ttacgccccgccctgcc-3') and subsequently ligated into the EcoRV restriction site between the *dnaK3* upstream and downstream regions in direct orientation. After transformation of the pILADnaK3 expression constructs and the *dnaK3* deletion construct into *Synechocystis* wild-type cells and selection of positive clones on plates containing increasing amounts of kanamycin (see above), complete segregation of the mutant strains was investigated by PCR.

**Miscellaneous techniques.** Sequences of the individual cyanobacterial DnaK proteins were retrieved from the Cyanobase (http://www.kazusa.or.jp/cyanobase/). The program CLUSTAL X (Thompson *et al.*, 1997) was used to calculate the alignments and the phylogenetic tree. All alignment parameters remained as pre-set by the program.
RESULTS

Three DnaK proteins in Synechocystis

In the genome of the cyanobacterium Synechocystis sp. PCC 6803 three genes code for potential DnaK proteins (Kaneko et al., 1996) (Fig. 1). The encoded DnaK proteins share a high degree of sequence homology to each other as well as to DnaK proteins from other species. The dnaK2 gene encodes a protein with a calculated molecular mass of 67 kDa and this protein shows the highest sequence identity to the E. coli DnaK protein as well as to chloroplast Hsp70 proteins. The DnaK1 protein has a calculated molecular mass of 75 kDa, and DnaK3 has a calculated molecular mass of 86 kDa. All three proteins share the common DnaK domain structure. The C-terminal region does not show a high degree of sequence similarity, but the C-terminal domains of Hsp70 proteins are in general not highly conserved. Based on the sequence analysis, all three Synechocystis DnaK proteins could fulfil similar physiological functions but it is not yet clear if all proteins are functionally expressed in Synechocystis and if the functions overlap in vivo.

Expression and localization of three DnaK proteins in Synechocystis

Expression of the three different DnaK isoforms was tested by Western blot analysis using antibodies specifically recognizing the individual proteins. Since the three DnaK proteins encoded in Synechocystis share a high degree of sequence similarity (Fig. 1) it was likely that an antibody raised against one protein also recognizes the other isoforms. Therefore, antibodies were raised against peptide stretches that are specific for one protein (underlined in Fig. 1). To test whether the generated antibodies recognize the individual DnaK proteins and are specific for one

Fig. 1. Sequence alignment of the three DnaK proteins from Synechocystis PCC 6803 and from E. coli. Peptide sequences that were used for raising antibodies against the individual Synechocystis DnaK proteins are underlined.
isoform, we heterologously expressed the three Synechocystis proteins in E. coli for further analyses. As can be seen in Fig. 2, all antibodies recognized the corresponding DnaK proteins, which were heterologously expressed in E. coli, and all antibodies specifically detected only a single DnaK protein. Since the antibodies showed a high degree of specificity and allowed detection of the individual DnaK proteins they were used to test the expression of the three DnaK proteins in Synechocystis cells. After separation of Synechocystis whole-cell extract by SDS-PAGE, all three DnaK isoforms were detected by Western blot analysis (Fig. 2). This observation proves that all three dnaK genes are functionally expressed in Synechocystis and suggested a specific physiological function for each protein.

To further determine the subcellular localization of the individual DnaK proteins, soluble proteins of Synechocystis wild-type cells were separated from membranes and the individual fractions were subjected to Western blot analysis (Fig. 3). While DnaK1 and DnaK2 were mainly detected in the soluble protein fraction, DnaK3 was almost exclusively found attached to Synechocystis membranes. Since for none of the three DnaK proteins has a signal anchor been predicted by computational methods we further investigated membrane attachment of the DnaK proteins by alkali extraction using carbonate as well as NaOH. As can be seen in Fig. 3, while the DnaK proteins can be totally extracted from membranes with NaOH, they remain almost completely in the pellet upon extraction with Na2CO3. These results suggest that all three DnaK proteins strongly bind to membranes although they lack any membrane-spanning domains.

Deletion of the dnaK genes

The observation that all three DnaK proteins are expressed in Synechocystis raised the questions if all three proteins are essential for viability, if the functions of the three proteins overlap and can be taken over by another protein, or if each DnaK protein fulfils a specialized function in vivo. To study if the individual proteins have specific or overlapping physiological functions, we attempted to delete the three genes individually in Synechocystis (Fig. 4a). A kanamycin resistance cassette was introduced into each dnaK gene on a plasmid and the deletion constructs were transformed into Synechocystis, which is naturally competent and integrates DNA into the chromosome via homologous recombination.

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Fig. 2. All three DnaK proteins are expressed in vivo in Synechocystis. The three DnaK proteins were heterologously expressed in E. coli ΔdnaK52. Equal protein amounts of cellular extract together with total extract of Synechocystis wild-type cells were subjected to Western blot analysis. The individual DnaK proteins were detected using the corresponding peptide antibody. Syn. CE, Synechocystis cellular extract.

Fig. 3. Cellular localization of the DnaK proteins from Synechocystis. Total cellular extract (CE) of Synechocystis wild-type cells was separated into soluble (S) and membrane protein (P) fractions. Membranes of a Synechocystis wild-type culture were incubated with buffer containing either 100 mM NaOH or 100 mM Na2CO3. Non-extracted proteins in the membrane pellet fraction (P) were separated from extracted proteins in the soluble fraction (S). Equal volumes of the corresponding fractions were used for Western analysis.
Synechocystis contains multiple genome copies, and to test if all copies of the dnaK genes were disrupted, we isolated genomic DNA of the mutant strains and performed analytical PCR and restriction analysis to examine the dnaK genomic regions (Fig. 4b). In the ΔdnaK1 strain the dnaK1 gene was completely deleted and, therefore, dnaK1 is not essential for the viability of Synechocystis cells. In contrast to the dnaK1 deletion strain, we still observed wild-type dnaK2 genomic copies in the ΔdnaK2 strain. This confirms that this gene cannot be completely deleted and thus fulfills an essential function under the tested growth conditions. Also in the case of the dnaK3 gene we still detected the wild-type dnaK3 fragment in the mutant strain, indicating that the gene cannot be completely replaced and is therefore essential for cell viability, as dnaK2 is. Furthermore, in the strains where some of the wild-type dnaK2 or dnaK3 genes have been disrupted, the amount of expressed DnaK2 proteins remained similar and the amount of DnaK3 was somewhat reduced when compared to the wild-type (Fig. 4c). In contrast, in the ΔdnaK1 strain we were no longer able to detect the DnaK1 protein, as expected. To test whether the disruption of dnaK1 has any impact on the Synechocystis cells, we characterized the mutant strain in parallel with the wild-type. Interestingly, we were not able to observe any changes of the phenotype with the ΔdnaK1 strain. Pigment content and growth rates were similar to wild-type cells, as was photosynthetic oxygen production and the relative amounts of photosystem 2 and photosystem 1 (data not shown). These observations suggest that DnaK1 has no essential function under any of the chosen growth conditions.
Complementation of an E. coli TS ΔdnaK strain

To test whether expression of the individual Synechocystis DnaK proteins complements a temperature-sensitive E. coli ΔdnaK strain, the individual Synechocystis dnaK genes, as well as the E. coli dnaK gene (control), were cloned into a pMal-c2-based expression plasmid. The individual plasmids were transformed into E. coli ΔdnaK756 and subsequently grown at 30 °C and 42 °C. As can be seen in Fig. 5(a), only expression of the E. coli DnaK protein restored growth of the temperature-sensitive E. coli ΔdnaK strain at 42 °C, whereas expression of none of the Synechocystis dnaK genes could complement the growth defect of the E. coli cells. Even when the growth temperature and/or the concentration of IPTG used for induction of the DnaK expression were varied, a functional complementation of the temperature-sensitive phenotype was never observed.

To further exclude that a disturbed dnaK expression had caused the missing complementation competence, total cell extract, soluble and membrane-bound proteins, as well as inclusion bodies were prepared and separated by SDS-PAGE. As can be seen in Fig. 5(b), all proteins were expressed in the temperature-sensitive E. coli strain but only DnaK2 was mainly expressed into the cytoplasm. DnaK1 as well as DnaK3 were found either deposited in inclusion bodies or attached to the E. coli cytoplasmic membrane. The subcellular localization of DnaK1 and DnaK3 could already explain the missing complementation after expression of these two proteins, as further discussed below.

The DnaK3 C terminus is essential

Whereas the nucleotide- and substrate-binding domains of the three Synechocystis DnaK proteins are highly homologous (Fig. 1), the proteins have highly diverse C termini and, furthermore, the C terminus of DnaK3 is extended, when compared to the two other DnaK proteins. Nimura et al. (1996) proposed that the C terminus of the DnaK3 protein from Synechococcus sp. PCC 7942 can be deleted without altering the phenotype of the generated strain when compared to the wild-type. This observation was unexpected since all cyanobacterial DnaK3 proteins appear to contain a prolonged C terminus, which strongly suggests an essential in vivo function of this domain. To analyse the potential function of the DnaK3 C-terminal extension in Synechocystis we tried to express in Synechocystis a truncated version of DnaK3 which lacks the last 78 amino acids. To this end, the full-length, as well as the truncated dnaK3 gene, were integrated into a neutral site of the Synechocystis genome, and both genes were controlled by the original dnaK3 promoter. After several rounds of selection the additional genes were completely integrated into the Synechocystis genome and we tested if expression of the full-length as well as of the truncated DnaK3 proteins at a different site of the Synechocystis chromosome facilitates complete deletion of the original dnaK3 gene. Therefore, we attempted to completely replace the original dnaK3 gene with the cat gene, which encodes the chloramphenicol acetyltransferase. After several rounds of selection on selective medium the dnaK3 gene was completely replaced in the strain in which the full-length dnaK3 gene was expressed from a different site on the chromosome (Fig. 6). This observation confirms that deletion of dnaK3 is possible when the gene is expressed from a different locus and that, therefore, the result shown in Fig. 4 was not caused by polar effects of the deletion construct on flanking genes. In contrast, expression of the C-terminally truncated dnaK3 gene from a different site of the Synechocystis chromosome did not result in complete replacement of the original dnaK3 gene. This observation proves that the C terminus of DnaK3 is absolutely essential under the chosen experimental conditions, and a truncated DnaK3 protein cannot fulfil the physiological functions of DnaK3.

Differential heat-shock response of DnaK proteins

The expression of DnaK2 from Synechocystis has previously been shown to be upregulated upon heat shock and several
other stresses like UV light, ethanol, salt and high light (Chitnis & Nelson, 1991; Fulda et al., 2006; Mary et al., 2004; Varvasovszki et al., 2003). Therefore, we addressed the question whether the three individual dnaK genes of Synechocystis are induced upon heat shock. To test this on a protein level, Synechocystis wild-type cells were shifted from 34 °C to 43 °C and samples were subsequently taken at various time points. Crude cell extracts were separated on SDS-gels and the amount of individual DnaK proteins was examined by Western blot analysis. As can be seen in Fig. 7(a), the protein levels of each DnaK protein irregularly increased and decreased during heat shock. A rapid increase of the protein amounts within the first 15 min after the temperature shift was observed followed by a decrease of the protein levels and a subsequent slow increase. Apparently, the DnaK2 protein level at 420 min is much higher than the level before heat stress, which also seems to be the case for the DnaK3 protein. To further support the unexpected observation that DnaK expression is not gradually increased after heat shock and to exclude experimental artefacts, we further tested the promoter activities of the three dnaK genes under heat stress to compare changes in the protein level with the promoter activities. The dnaK promoter regions were fused to a bacterial luxAB reporter gene as described in Methods, and specific Synechocystis reporter strains were generated. An analysis of the promoter activities of the three dnaK reporters under photoautotrophic growth conditions demonstrated that the dnaK2 promoter shows the highest activity, followed by the dnaK3 promoter (Fig. 7b). The dnaK1 promoter was found to be not strongly but significantly active, which further supports the apparent detection of DnaK1 by Western analyses (Fig. 7a). The dnaK reporter strains generated were subsequently shifted to higher temperatures and the individual promoter activities were determined at various time points to follow changes in the promoter activities upon heat stress. The activities of the individual dnaK promoters were set as one at time point zero and all changes are shown relative to the activities before heat stress. As can be seen in Fig. 7(c), the activities of all three dnaK promoters showed a rapid increase during the first 15 min after shifting the cultures to the higher temperature. However, while the promoter activities of dnaK1 and dnaK3 only slightly increased (about twofold), the dnaK2 promoter activity was already significantly higher at this stage and reached its maximum after 30–45 min. In all three cases, the promoter activity levels rapidly declined after the initial fast increase before they slowly increased again. These observations strongly support the data obtained by Western blot analysis. Based on the promoter activity measurements, only the dnaK2 promoter is significantly upregulated upon heat shock and stays above the original level during the entire period at higher temperature. This upregulation can also be observed on the DnaK2 protein level by Western analysis (Fig. 7a). On the protein level also DnaK3 synthesis seemed to be slightly increased during heat shock, which was not observed in the promoter activity measurement, but the amount of DnaK proteins might also be regulated on the translational level or on the level of protein degradation.

**DISCUSSION**

**On the existence of multiple DnaK proteins in cyanobacteria**

After annotation of the first completely sequenced genome of a cyanobacterium it became evident that several proteins are encoded by gene families in Synechocystis sp. PCC 6803 (Kaneko et al., 1996; Kaneko & Tabata, 1997). The probably best characterized families are the psbA1–3 and the petCl–3 families in Synechocystis (Mate et al., 1998; Mohamed et al., 1993; Salih & Jansson, 1997; Schneider et al., 2002, 2004a, b). Obviously, the potential to encode a protein by various genes has proven evolutionarily successful, and it has been speculated that the possibility to regulate an interplay between various genes and proteins could be one way for cyanobacteria to respond to different environments and to environmental changes (Schneider & Schmidt, 2005). This assumption would imply that the proteins fulfil identical, or at least very similar, functions in vivo, as has been observed for example for members of the PsbA protein family (Mohamed et al., 1993; Salih & Jansson, 1997). But why do cyanobacteria need three DnaK
proteins when other bacteria have only one and even this is dispensable?

So far, several cyanobacterial genomes have been completely sequenced and in all these genomes several DnaK proteins are encoded. Based on a computational analysis, these proteins can clearly be clustered in three distinct groups, representing the three individual DnaK proteins (Fig. 8). It is noteworthy that the DnaK2 branch shows the highest sequence homology to other DnaK proteins from bacteria and chloroplasts.

The observation that in all cyanobacteria examined a DnaK protein family exists indicates that the existence of more than one DnaK protein is of physiological importance, especially for these bacteria. As shown in Fig. 1, the amino acid sequences of the three _Synechocystis_ DnaKs are highly conserved and the mainly observed differences are the C-terminal extensions of the protein. However, sequence analysis of these extensions does not allow any conclusions to be drawn about the potential functional differences of the proteins. It is, nevertheless, striking that all DnaK3 proteins have an extra C-terminal extension and in all available DnaK3 sequences a motif rich in tryptophan and aspartic acid can be found at the very C-terminal end of this domain (compare Fig. 1).

**Expression and subcellular localization of DnaKs**

One major goal of this study was to answer the question if all three DnaK proteins are functionally expressed in _Synechocystis_. Based on Western analyses it has been observed that three DnaK proteins are expressed in the cyanobacterium _Synechococcus PCC 7942_ (Nimura et al., 2001), whereas it has recently been proposed that only DnaK2 is expressed in _Synechocystis_ (Varvasovszki et al., 2003). But since the existence of a DnaK protein family is

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**Fig. 7.** Heat-shock response of DnaK expression in _Synechocystis_. (a) Influence of heat stress on DnaK protein levels. An exponentially growing _Synechocystis_ wild-type culture was divided into 100 ml culture samples and shifted from 34 °C to 43 °C. Samples were taken at the indicated time points. Equal protein amounts of total cellular extracts were subjected to Western blot analysis. (b) Relative promoter activities of the three dnaK genes. The promoter regions of the three individual dnaK genes were fused to the bacterial luxAB reporter gene as described in Methods. Promoter activities were measured for 30 s after addition of 1 mM decanal. As a control a _Synechocystis_ reporter strain was constructed in which no promoter was fused to the reporter gene (background fluorescence). (c) Promoter activity changes of the dnaK genes during heat shock. Cultures (100 ml) of the dnaK promoter-probe strains at an OD$_{750}$ of 0.5 were shifted to 43 °C. Samples were taken for luminescence measurement at the indicated time points. Mean values of three independent measurements were normalized to the background values of the control strain. All activities are shown relative to the activity at time point 0 (before temperature shift), which was set as 1.
conserved in cyanobacteria it appeared likely that also in *Synechocystis* all three proteins are expressed and active. To address this question we performed Western blot analysis and we could show that in *Synechocystis* clearly all three DnaK proteins were recognized by the specific antibodies (Fig. 2), which is in agreement with the situation in *Synechococcus* (Nimura et al., 2001). It was, however, possible that the discrepancy between this study and that of Varvasovszki et al. (2003) was based on different growth conditions, which may have resulted in different expression of the three *dnaK* genes. However, besides the expression of DnaK2, which has been detected in several studies, in a recent proteomic shotgun approach also DnaK3 has been detected (Gan et al., 2005), and also the DnaK1 protein has been found in a proteomic study of salt stress response in *Synechocystis* (Fulda et al., 2006). Regardless of the growth conditions we were able to detect the expression of all three DnaK proteins in *Synechocystis*, and at this stage we cannot explain the different observations made here and by Varvasovszki et al. (2003). Based on our results we conclude that three DnaK proteins are constitutively expressed in *Synechocystis*, and, under normal growth conditions, the *dnaK2* promoter has the highest activity followed by the *dnaK3* and *dnaK1* promoters (Fig. 7b). When compared to the other two, the *dnaK1* promoter has a weak but significant activity and the DnaK1 protein was also clearly detected in Western blot analyses (Fig. 2).

Analysis of the subcellular localization of the DnaK proteins showed that while DnaK1 and DnaK2 can mainly be found in *Synechocystis* soluble cell extract, DnaK3 was only found attached to *Synechocystis* membranes and no protein was found in the soluble protein fraction. In *Synechococcus* DnaK3 is bound to thylakoid membranes, and it was further suggested that the protein is bound via the peptide-binding domain to the membrane (Nimura et al., 2001).
type the observation that even after replacement of some wild-type deletion on adjacent genes can be excluded. Furthermore, dnaK3 strain’s phenotype (Fig. 6), polar effects of the site of the gene. However, since expression of DnaK3 from a different dnaK3 deletion of the original gene is organized in a gene cluster with a dnaJ gene. The two encoded proteins are distinct physiological functions in vivo. In the two cyanobacteria Synechococcus PCC 7942 and Synechocystis PCC 6803 three DnaK proteins are functionally expressed, which further supports this assumption. In line with a proposed important function we were not able to delete the dnaK2 and dnaK3 genes. The two encoded proteins are therefore essential in Synechocystis and have specific, yet not redundant, functions. If the functions of these two proteins overlapped one would have expected that the genes could be deleted individually but not in combination. Since even the deletion of a single gene was not possible, both proteins seem to have a distinct physiological role, which is further supported by the observed differences in the subcellular localization. However, gene disruption was perhaps not possible due to polar effects of the intended deletion on flanking genes. Based on a sequence analysis, dnaK2 is not organized in an operon, whereas dnaK3 is organized in a gene cluster with a dnaJ gene. However, since expression of DnaK3 from a different site of the Synechocystis chromosome allowed complete deletion of the original dnaK3 gene without altering the strain’s phenotype (Fig. 6), polar effects of the dnaK3 deletion on adjacent genes can be excluded. Furthermore, the observation that even after replacement of some wild-type dnaK3 genes the DnaK3 protein level was not dramatically altered (Fig. 4) strongly suggests that this protein is crucial. In contrast, after deletion of dnaK1 the mutant’s phenotype was indistinguishable from the wild-type. Since DnaK1 is expressed in wild-type cells, it is very likely that it has a physiological function, which either has not been identified yet or can be taken over by any of the two remaining DnaK proteins. But what could be the physiological functions of three DnaK proteins in Synechocystis?

The DnaK2 protein from Synechococcus PCC 7942 functionally complemented a temperature-sensitive ΔdnaK E. coli strain, whereas the two other Synechococcus DnaK proteins could not (Nimura et al., 2001). All attempts to complement a temperature-sensitive E. coli ΔdnaK strain with the DnaK proteins from Synechocystis failed and even the DnaK2 protein from Synechocystis could not complement the defect. This result should, however, be taken with caution since it does not clearly show that the three proteins do not have DnaK-like functions. In the case of DnaK3 the missing complementation was not surprising since the protein was only found in inclusion bodies after expression in E. coli. Therefore, the protein could most likely not fulfill the function of a cytoplasmic Hsp70. Since DnaK proteins in general work in a concerted mechanism with co-chaperones (GrpE and DnaJ), it is also possible that the individual DnaK proteins from either Synechocystis or Synechococcus cannot functionally interact with the co-chaperones from E. coli, and it has indeed already been shown that co-expression of a DnaJ protein is needed to complement the E. coli ΔdnaK defect with proteins from a different organism (Mogk et al., 1999). A co-expression of the Synechocystis DnaK proteins with a DnaJ protein is, however, not straightforward. In the genome of Synechocystis several DnaJ proteins are encoded (Kaneko et al., 1996). It is very likely that the individual functions of the three DnaK proteins are further defined by distinct interactions with individual DnaJ proteins, and a major task of our future work is to describe this functional network. Besides interactions of DnaK proteins with co-chaperones it has become evident in recent years that also individual Hsp70 proteins can functionally interact (Steel et al., 2004; Yam et al., 2005). Therefore, differential interactions between individual DnaK proteins from Synechocystis could also determine their physiological functions.

Physiological functions of multiple DnaK proteins

The observation that a dnaK gene family is common in cyanobacteria indicates that the individual proteins have distinct physiological functions in vivo. In the two cyanobacteria Synechococcus PCC 7942 and Synechocystis PCC 6803 three DnaK proteins are functionally expressed, which further supports this assumption. In line with a proposed function where we were not able to delete the dnaK2 and dnaK3 genes. The two encoded proteins are therefore essential in Synechocystis and have specific, yet not redundant, functions. If the functions of these two proteins overlapped one would have expected that the genes could be deleted individually but not in combination. Since even the deletion of a single gene was not possible, both proteins seem to have a distinct physiological role, which is further supported by the observed differences in the subcellular localization. However, gene disruption was perhaps not possible due to polar effects of the intended deletion on flanking genes. Based on a sequence analysis, dnaK2 is not organized in an operon, whereas dnaK3 is organized in a gene cluster with a dnaJ gene. However, since expression of DnaK3 from a different site of the Synechocystis chromosome allowed complete deletion of the original dnaK3 gene without altering the strain’s phenotype (Fig. 6), polar effects of the dnaK3 deletion on adjacent genes can be excluded. Furthermore, the observation that even after replacement of some wild-type dnaK3 genes the DnaK3 protein level was not dramatically altered (Fig. 4) strongly suggests that this protein is crucial. In contrast, after deletion of dnaK1 the mutant’s phenotype was indistinguishable from the wild-type. Since DnaK1 is expressed in wild-type cells, it is very likely that it has a physiological function, which either has not been identified yet or can be taken over by any of the two remaining DnaK proteins. But what could be the physiological functions of three DnaK proteins in Synechocystis?

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It appears, however, already safe to argue that the DnaK3 C terminus is essential for the physiological function of the protein, since a C-terminally truncated DnaK3 version cannot substitute for the full-length protein in vivo (Fig. 6). Surprisingly, it has been reported that a C-terminally truncated DnaK3 protein from Synechococcus PCC 7942 can substitute for the wild-type protein without affecting the phenotype of the cyanobacterial strain (Nimura et al., 1996). While in this study only the last 78 amino acids of DnaK3 were deleted, in the study of Nimura et al. (1996) the last 150 residues were deleted, and this truncation should affect the physiological function of the protein even more severely. At this stage we cannot explain these
different physiological observations but the disparity may indicate physiological differences between the in vivo function of DnaK3 in the two cyanobacterial strains.

**Different heat-shock responses of the three DnaK proteins**

After transferring *Synechocystis* cells from 34 °C to 43 °C we observed changes in the protein levels for all three DnaK proteins. In *Synechococcus* it has been described that the steady-state level of DnaK3 did not change after heat shock whereas the protein levels of DnaK1 and DnaK2 did change. The DnaK1 protein level decreased after 120 min of heat stress whereas the DnaK2 level was slightly increased (Nimura *et al.*, 2001). As can be seen in Fig. 7, we observed an initial decrease of the DnaK1 and DnaK3 protein levels after growing the cells under heat stress, but both protein levels reached the wild-type level again after 420 min. In contrast, we observed a strong DnaK2 overexpression in *Synechocystis* after heat shock. A stress-induced increase of DnaK2 expression in *Synechocystis* has been observed on both the RNA and protein level (Choi *et al.*, 2000; Fulda *et al.*, 2006; Huang *et al.*, 2006; Mary *et al.*, 2004; Varvasovszki *et al.*, 2003), but the stress response was only monitored at one time point after stress. Interestingly, after a rapid initial increase the DnaK2 protein level decreased slightly followed by a continuous accumulation of DnaK2. A similar situation was also observed for DnaK1 and DnaK3, although the changes were not as pronounced as in the case of DnaK2. To exclude experimental artefacts we further constructed *Synechocystis* reporter strains, in which the promoters of the individual *dnaK* genes control the expression of the reporter. When these strains were used to follow promoter activities of the three *dnaK* genes we found a similar pattern as observed by Western analysis (Fig. 7c). All three *dnaK* genes showed an increased promoter activity followed by an activity decrease. The *dnaK1* and *dnaK3* genes thereafter slowly reached the same expression level as before heat stress; these observations support the data obtained on the protein expression level. After the initial strong increase in the *dnaK2* promoter activity also the activity of this promoter markedly decreased, although it still remained above the initial level. The *dnaK2* promoter activity then increased gradually, as observed on the protein level. Recently, the heat-stress response of various genes has been followed in *Synechocystis* and expression of the *dnaK2* gene has been tested on the mRNA level 20 and 60 min after heat stress (Suzuki *et al.*, 2006). In this study accumulation of *dnaK2* mRNA was more than 20-fold increased after 20 min but was only twofold increased after 60 min, when compared to the mRNA level prior to heat stress, and this is in excellent agreement with the finding of this study.

The observation that the promoter activities of all three *dnaK* genes, and especially of *dnaK2*, strongly increase after heat shock but thereafter markedly decrease is surprising and can most likely not be explained directly by the function of the DnaK proteins. Besides the DnaK protein and the associated co-chaperones, σ32 factors have important roles in heat-stress response in bacteria (for a recent review see Hengge-Aronis, 2002). *E. coli* DnaK functionally interacts with the σ32 factor and is involved in the regulation of its translation as well as in regulation of σ32 proteolysis. It is possible that the initial strong heat-shock response of the *dnaK2* gene (Fig. 7) somehow negatively regulates the activity of the σ32 factor, resulting in downregulation of *dnaK* expression. But such an assumption has to be made with caution in the absence of any experimental evidence, and we are currently investigating the regulation of *dnaK2* expression in more detail.

In general, the observation that a stress response can strongly vary at various time points suggests that studies which determine stress responses at only one time point after induction have to be interpreted with caution. The observed DnaK2 heat stress response was highly dependent on the selected time point after heat stress. The response of the expression of a protein to any external stimuli should always be followed over a period of time to exclude the possibility that a transient activity minimum or maximum is measured.

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