Specific and promiscuous functions of multiple DnaJ proteins in *Synechocystis* sp. PCC 6803

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Cyanobacterial genomes typically encode multiple Hsp70 (DnaK) and Hsp40 (DnaJ) chaperones, and in the genome of the cyanobacterium *Synechocystis* PCC 6803, three DnaK proteins are encoded together with seven DnaJ proteins. While only two of the DnaJ proteins can complement the growth defect of an *Escherichia coli* Δ dnaJ strain, only disruption of the dnaJ gene *sll0897* resulted in a growth defect at elevated temperatures. Based on the domain structure and the phenotype observed following disruption of the encoding gene, *Sll0897* can be classified as a canonical heat-shock protein in *Synechocystis*. Furthermore, most dnaJ genes could be deleted individually, whereas disruption of the gene encoding the DnaJ *Sll1933* failed, which suggests an essential, yet undefined, function for *Sll1933*. Since after deletion of the remaining dnaJ genes the phenotypes were not altered, the functions of these DnaJs either are not critical or are taken over by the remaining DnaJs. Nevertheless, only the two dnaJ genes *sll0909* and *sll1384* could be disrupted in combination, suggesting physiological functions for the two encoded proteins which either are not overlapping and/or can be fulfilled by the remaining DnaJs in the double-disruption strain. Taken together, the present analysis indicates specific and promiscuous functions for multiple DnaJ proteins in *Synechocystis*.

INTRODUCTION

Molecular chaperones of the Hsp70 (heat-shock proteins of 70 kDa) family are involved in diverse fundamental cellular processes in pro- and eukaryotes, such as the *de novo* folding of newly synthesized proteins, the prevention of protein misfolding and aggregation, protein transport and translocation, as well as the control of regulatory protein functions (Mayer & Bukau, 2005). The intrinsic ATPase activity of DnaK, which results in tight binding of an associated protein substrate (Mayer & Bukau, 2005), is typically low, and thus ATP hydrolysis is assisted by a DnaJ co-chaperone. The prototypic DnaJ protein of *Escherichia coli* has a molecular mass of ~40 kDa, and DnaJ-like chaperones therefore belong to the class of Hsp40s. Hsp40s can stimulate ATP hydrolysis of Hsp70s more than 1000-fold (Laufen *et al.*, 1999). Interaction between DnaJ and DnaK leads to an accelerated hydrolysis of ATP, which results in closing of the DnaK peptide-binding domain (Genevaux *et al.*, 2007) and tight binding of the substrate. Subsequently, the nucleotide exchange factor GrpE interacts with DnaK, resulting in an exchange of ADP for ATP and dissociation of the bound substrate.

After independently binding a substrate (Szabo *et al.*, 1996), DnaJ transfers a protein substrate to the C-terminal peptide-binding domain (PBD) of DnaK (Acebrón *et al.*, 2008; Gamer *et al.*, 1996; Greene *et al.*, 1998; Han & Christen, 2003; Laufen *et al.*, 1999). DnaJ–DnaK interactions are mediated by the structurally conserved N-terminal J domain of DnaJs, which is absolutely essential for DnaJ activity (Wall *et al.*, 1994). While the amino acid sequences of J domains are not highly conserved, this domain typically consists of about 70 aa, which form four α -helices (Szyperski *et al.*, 1994). In the loop region between helices II and III, a characteristic histidine-proline-aspartate amino acid motif (HPD motif) is conserved, which is essential for stimulating the Hsp70 ATPase activity (Szyperski *et al.*, 1994; Tsai & Douglas, 1996; Wall *et al.*, 1994). DnaJ proteins contain additional domains next to the J domain, and the domain structure is the basis for a historical classification of DnaJ proteins into three types (Cheetham & Caplan, 1998). The *E. coli* DnaJ protein is the canonical representative of a type I DnaJ protein, which contains the N-terminally located J domain followed by a glycine- and phenylalanine (GF)-rich region, a cysteine-rich region characterized by repeats of the conserved CxxCxGxG motif, and the C-terminal domain (CTD). The cysteine-rich region and the CTD are involved

Abbreviations: CTD, C-terminal domain; PRP, pentapeptide repeat; TPR, tetratricopeptide repeat; wt, wild-type.

in substrate binding by DnaJ (Lu & Cyr, 1998; Szabo *et al.*, 1996). In contrast to type I DnaJ proteins, type II proteins do not contain the cysteine-rich region, whereas type III proteins only contain a J domain. DnaJ proteins of types I and II may fulfil similar functions and bind independently to non-native substrates, whereas type III DnaJs are not considered to be independent molecular chaperones (Kelley, 1998).

It appears to be common to most organisms that multiple DnaJ/Hsp40 proteins work together with a limited number of DnaK/Hsp70 proteins in organelles or subcellular compartments. Specific interactions of Hsp70 proteins with defined Hsp40 co-chaperones permit the control of the function and specificity of Hsp70 proteins. In eukaryotes, typically multiple Hsp70 chaperones exist as well as many Hsp40 co-chaperones. In *Saccharomyces cerevisiae*, three cytosolic Hsp70s coexist with no fewer than 14 Hsp40 proteins, and in total at least 10 Hsp70s and 22 Hsp40s are found in the various compartments of the cell (Walsh *et al.*, 2004). In the *E. coli* genome, six DnaJ-like proteins are encoded (Blattner *et al.*, 1997), and the individual DnaJ proteins interact with specific DnaK-like proteins (Genevaux *et al.*, 2007). Cyanobacterial genomes typically encode multiple DnaK and DnaJ proteins. The diversity of these heat-shock proteins has been studied to some extent in the cyanobacteria *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803 (Nimura *et al.*, 2001; Rupprecht *et al.*, 2007, 2008, 2010; Sato *et al.*, 2007). In the genomes of both *Synechococcus* and *Synechocystis*, three DnaK proteins are encoded (Holtman *et al.*, 2005; Kaneko *et al.*, 1996). While the exact physiological functions of multiple DnaK proteins in cyanobacteria are still enigmatic, only DnaK2 and DnaK3 are essential in *Synechocystis* and *Synechococcus* (Nimura *et al.*, 2001; Rupprecht *et al.*, 2007). Accordingly, DnaK2 seems to be the major Hsp70 protein involved in the stress response (Rupprecht *et al.*, 2008), whereas DnaK3 has a specialized function, which involves the highly conserved prolonged C terminus of the protein (Rupprecht *et al.*, 2010).

Studies of the functions of four out of the (at least) 10 DnaJ-like proteins encoded in the genome of the cyanobacterium *Synechococcus* sp. PCC 7942 have indicated that two DnaJ proteins are upregulated upon heat and high-light stress (Sato *et al.*, 2007). Furthermore, one DnaJ protein appears to specifically interact with the *Synechococcus* DnaK2 protein (Watanabe *et al.*, 2007). Unfortunately, so far no further functional analysis of the remaining DnaJ proteins of *Synechococcus* has been performed, and thus a complete picture of the features and functions of the multiple cyanobacterial DnaJs and their interactions with DnaKs has yet to emerge.

In the present study we have investigated the features and functions of the *Synechocystis* sp. PCC 6803 DnaJ proteins in more detail. Only expression of two *dnaJ* genes in a *dnaJ*-deficient *E. coli* strain complemented the growth phenotype of the *E. coli* deletion strain at elevated

temperatures, which indicates that (at least) the two encoded proteins represent classical DnaJs. To further analyse the function of multiple DnaJ proteins in the cyanobacterium *Synechocystis*, all *dnaJ* genes were disrupted individually as well as in combination. Based on this analysis, only the DnaJ protein encoded by the gene *sll1933* appears to be essential in *Synechocystis*, which indicates that only Sll1933 has a specialized function, whereas the remaining DnaJ proteins have partly overlapping functions and can substitute for one another. However, this replaceability is limited, since only two *dnaJ* genes could be deleted in combination. Furthermore, deletion of the *dnaJ* gene *sll0897*, which encodes the only prototypical type I DnaJ protein, resulted in impaired growth of *Synechocystis* under cold- and heat-stress conditions. Our analyses suggest that the type I DnaJ protein Sll0897 is a genuine Hsp40 heat-shock protein in *Synechocystis*, whereas only the DnaJ protein Sll1933 has an essential, yet undefined, function.

METHODS

Cell culture. *Synechocystis* sp. PCC 6803 wild-type (wt) was cultivated in liquid BG11 medium (Rippka *et al.*, 1979) at 34 °C under 33 $\mu\text{E m}^{-2} \text{s}^{-1}$ of fluorescent cold white light. For cultivation of mutant strains, antibiotics were added to the medium. All cultures were grown photoautotrophically. *E. coli* strain DH5 α was used for plasmid propagation and was grown in Luria–Bertani (LB) medium. The temperature-sensitive *E. coli* ΔdnaJ strain OD259 (Kelley & Georgopoulos, 1997) was cultivated in LB medium at 30 °C.

Disruption of *dnaJ* genes. All molecular methods were done following standard procedures (Sambrook & Russell, 2001). For disruption, individual *dnaJ* genes were amplified by PCR using the primers listed in Table 1 and genomic *Synechocystis* wt DNA as template.

PCR fragments were ligated directly into the plasmid pCR2.1-TOPO (Invitrogen), and a chloramphenicol-resistance cassette (*cat*), derived from plasmid pACYC184 (New England Biolabs), was inserted into the genes as described in detail below. The constructs were transformed into *Synechocystis* wt cells (Williams, 1988) and positive clones were selected on BG11 agar plates containing 10 μg chloramphenicol ml^{-1} . Growing cells were continuously transferred onto selective agar plates containing increasing concentrations of chloramphenicol (up to 150 $\mu\text{g ml}^{-1}$) for more than 1 year.

In addition, the respective pCR2.1-*dnaJ* plasmids (compare above) were used to interrupt the individual *dnaJ* genes by a spectinomycin-resistance cassette (*spec*) derived from plasmid pSpec (Prentki & Krisch, 1984), as described for the *cat* cassette. Individual plasmids were transformed into *Synechocystis* ΔdnaJ strains, and positive clones were initially selected on BG11 agar plates containing 30 μg chloramphenicol ml^{-1} and 10 μg spectinomycin ml^{-1} . Positive clones were continuously transferred onto selective agar plates containing increasing concentrations of spectinomycin (up to 230 $\mu\text{g ml}^{-1}$) for more than 1.5 years. To check whether the deletion strains were completely segregated, genomic DNA was isolated from strains grown for at least three generations in antibiotic-free medium, and the corresponding *dnaJ* genes were analysed by PCR using the primers listed in Table 1.

Complementation of an *E. coli* temperature-sensitive ΔdnaJ strain. The *E. coli* *dnaJ* gene was amplified from genomic DNA by

Table 1. Primers used in this study

Gene	Sequence (5'→3')	
	5' Primer	3' Primer
<i>slr0093</i>	ataggccatagggcatcaacagatttcaaag	atacgggatccttatgccaacaaattggcg
<i>sll0897</i>	ataggccatagctggggattattaccaaac	atacgaagctttattatggataatccccctaaaaatc
<i>sll0909</i>	ataggccatagggacacacacacacacac	atacgaagctttaaacccctaattgtcttttaaatc
<i>sll1011</i>	ataggccatagcaagagtttccactattac	atacgaagctttaactatgcttagtgccatcg
<i>sll1384</i>	ataggccatagagctcgtttcccaacaag	atacgaagctttattcttttggcccccgc
<i>sll1666</i>	atatgccatagggcccttctccttttgc	cgtgcctcgagtagttgatgaaccatggtaac
<i>sll1933</i>	ggcaaccctatggacaagtgcggaattattac	cggcaaaagtcttcttgattttaagcaattgtt
<i>dnaJEC</i>	ccgcatatggctaagcaagattattacg	gccggatccttagcgggtcaggtcgtc

PCR using the *dnaJEC* primers (Table 1). All *dnaJ* genes were expressed from a pACYC184-based expression plasmid (New England Biolabs), into which the *lac* promoter region from plasmid pMalp2 (New England Biolabs) was ligated. Therefore, the *MscI/HindIII* fragment from pMalp2 was ligated to the *HincII/HindIII* fragment of pACYC184, containing the *cat* cassette and the origin of replication. The genes encoding the single *E. coli* DnaJ and the individual *Synechocystis* DnaJ proteins (see above) were cloned into the *NdeI/BamHI* restriction-digested pACYC184 expression plasmid. The resulting plasmids were transformed into the *E. coli* Δ *dnaJ* strain OD259 (Kelley & Georgopoulos, 1997), and cells were grown at 30 °C in selective LB medium (with 20 μ g ml⁻¹ kanamycin and chloramphenicol) to OD₆₀₀ 0.5. The cultures were then diluted in LB medium from 10⁻² to 10⁻⁶, and 2 μ l was dropped on a selective LB agar plate. The plates were incubated overnight at the indicated temperatures.

After growing at 30 °C, transformed *E. coli* cells were also harvested and broken by sonication in 50 mM phosphate buffer, pH 8.0, containing a proteinase inhibitor cocktail (Sigma). MgCl₂ (2 mM) was added to the cellular extracts, and after addition of 1 mg ml⁻¹ DNase and RNase, cells were incubated for 30 min at room temperature. Extracts were subsequently centrifuged at 40 000 *g* and 4 °C for 1 h to separate membranes from soluble proteins. Ten micrograms of the soluble proteins were separated on SDS gels

(Laemmli, 1970), and gels were blotted onto a PVDF membrane for subsequent Western blot analyses. Heterologously expressed DnaJ proteins were used to raise antibodies against the individual DnaJ proteins in rabbits. Antisera (Gramsch Laboratories) were used at a 1:2000 dilution. The goat anti-rabbit secondary antibody was conjugated to peroxidase (Sigma) and used at a 1:30 000 dilution. A mouse antibody directed against the *E. coli* DnaJ protein (Pierce) was used at a 1:100 dilution.

RESULTS

At least seven DnaJ proteins are encoded in the *Synechocystis* genome

While in the genome of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 12 proteins possessing a J domain are encoded (Kaneko *et al.*, 1996), only seven contain a canonical J domain with the highly conserved HPD motif (Fig. 1). Based on the domain structure, these seven proteins can be classified into the three DnaJ types (Fig. 1). Only protein Sll0897 contains all domains typical of a type I DnaJ protein with an N-terminal J domain,

Fig. 1. Domain structure of the different *Synechocystis* DnaJ proteins and their classification into the three types of DnaJ proteins. For details see main text. Abbreviations: G/F, glycine/phenylalanine-rich region; CYS, cysteine-rich region; TM, transmembrane domain.

followed by the glycine/phenylalanine-rich region, a cysteine-rich motif and the CTD. Slr0093 and Sll1933 are type II DnaJ proteins and lack the cysteine-rich domain. The other four J domain proteins, Sll0909, Sll1011, Sll1384 and Sll1666, contain only the conserved J domain, and thus represent type III DnaJ proteins. However, these proteins exhibit additional characteristic domains, such as a pentapeptide repeat (PRP), a tetratricopeptide repeat (TPR) motif and a transmembrane domain, as further discussed below.

To test whether the individual *Synechocystis* DnaJ proteins can replace the function of the *E. coli* DnaJ, we expressed the *Synechocystis* *dnaJ* genes in the temperature-sensitive *E. coli* Δ *dnaJ* strain OD259 (Fig. 2). The OD259 strain is only able to grow at elevated temperatures if a functional *dnaJ* protein is expressed. *E. coli* cells transformed with the empty plasmid (–) grew only at 30 and 37 °C, whereas at 40 °C no growth was observed. In contrast, after expression of the wt *dnaJ* from *E. coli* (+), the strain grew well at 40 °C. Expression of the *Synechocystis* *dnaJ* genes in OD259 resulted in diverse growth behaviours. Only heterologous expression of the *Synechocystis* *dnaJ* genes *slr0093* and *sll0897* in the Δ *dnaJ* *E. coli* strain permitted growth at 40 °C. To further confirm expression of the individual *Synechocystis* DnaJ proteins in *E. coli*, soluble *E. coli* proteins were separated by SDS-PAGE and expression of the individual DnaJs was analysed by Western blotting. As

can be seen in Fig. 2(b), most of the DnaJ proteins from *Synechocystis* were expressed in *E. coli*, albeit to different extents. Only the *Synechocystis* DnaJ protein Sll1666 was not detected after induction of protein expression. While the expression level of the individual DnaJs differed in some cases, the results of the complementation assay were always as shown in Fig. 2(a), even when *E. coli* OD259 cells transformed with the various expression plasmids were grown in the presence of different IPTG concentrations. Together, these observations indicate that (at least) the *Synechocystis* *dnaJ* genes *slr0093* and *sll0897* encode proteins with a function homologous to that of the canonical *E. coli* DnaJ protein.

Sll1933 is the sole essential DnaJ protein in *Synechocystis*

The existence of multiple DnaJ proteins in *Synechocystis* raises the question of their specific and potentially overlapping functions. To study possible unique, essential or overlapping functions, we aimed to interrupt all *dnaJ* genes individually in *Synechocystis* wt cells. A chloramphenicol-resistance cassette (*cat*) was introduced into each of the seven canonical *Synechocystis* *dnaJ* genes, as illustrated in Fig. 3(a), and *Synechocystis* PCC 6803 wt was transformed using the deletion constructs. *Synechocystis*

Fig. 2. Complementation of the temperature-sensitive *E. coli* Δ *dnaJ* strain OD259. The individual *Synechocystis* *dnaJ* genes were expressed from a plasmid as described in Methods. (a) Cells were spotted on selective agar plates at various dilutions and incubated at 30, 37 or 40 °C. OD259 transformed with the empty plasmid (pACYC*lac*) served as a negative control (–); OD259 transformed with a plasmid expressing the *E. coli* *dnaJ* gene served as a positive control (+). (b) Soluble proteins (10 µg) from the *E. coli* cells analysed in (a) were separated on an SDS gel and expression of the individual DnaJ proteins was monitored by Western blot analyses. *E. coli* cells transformed with the respective expression plasmids (DnaJ) as well as the *E. coli* Δ *dnaJ* strain (Δ) were analysed together with 1 µg of the purified DnaJ proteins (+). The heterologously expressed and purified *Synechocystis* DnaJs contain an N-terminal decahistidine tag, and thus the molecular mass of the proteins is slightly increased. In the case of the *E. coli* DnaJ, soluble proteins from *E. coli* DH5 α were used as a positive control (+). The asterisk marks the position of the Sll1933 protein band in the transformed Δ *dnaJ* *E. coli* strain. Only the relevant regions of the blots are shown.

contains multiple identical genome copies, and to ensure that the *cat* cassette integrated in all respective *dnaJ* copies, cells were cultured for more than 1 year on selective agar plates with increasing chloramphenicol concentrations. To check whether the wt gene was disrupted in all DNA copies, we isolated genomic DNA of the putative deletion strains and amplified the individual *dnaJ* gene by PCR (Fig. 3b), since the size of the PCR fragment was increased by about 1000 bp when the *cat* gene was introduced. As controls, the individual wt *dnaJ*s were amplified from *Synechocystis* wt DNA, and the interrupted genes were amplified from the plasmids containing the respective interrupted *dnaJ* genes. As can be seen in Fig. 3(b), in the Δ *dnaJ* strains Δ *slr0093*, Δ *sll0897*, Δ *sll0909*, Δ *sll1011*, Δ *sll1384* and Δ *sll1666*, the respective *dnaJ* gene was completely disrupted. No PCR fragments having a size corresponding to the respective wt *dnaJ* gene were amplified by PCR, merely fragments having a

size corresponding to the interrupted *dnaJ* genes. It was only when using genomic DNA isolated from the putative Δ *sll1933* strain that we observed the wt *dnaJ* fragment of about 1000 bp after PCR as well as the *sll1933* fragment with the introduced resistance cassette (~2000 bp).

Taken together, the results indicate that only expression of *sll1933* is absolutely essential for the viability of *Synechocystis* cells.

Deletion of *sll0897* results in temperature-sensitivity

While in *Synechocystis* only *sll1933* appears to be absolutely essential for cell growth and/or survival, deletion of the other *dnaJ* genes could result in growth defects, as for example observed following deletion of the canonical *E. coli* *dnaJ* gene (Georgopoulos *et al.*, 1980). Therefore, we

Fig. 3. Disruption of *Synechocystis* *dnaJ* genes. (a) Strategy for interruption of the individual *dnaJ* genes in the genome of *Synechocystis* with a chloramphenicol-resistance cassette (*cat*). Restriction sites used for integration of the resistance cassette into the gene are indicated. Arrows indicate the binding sites of the primers used for the segregation control. The size of each *dnaJ* is given in parentheses. (b) PCR analyses of the generated *Synechocystis* Δ *dnaJ* strains. The individual *dnaJ* region was amplified from the respective mutant strain (Δ). As controls, the wt *dnaJ* gene was amplified from genomic wt DNA (+) and the interrupted gene was amplified from the plasmid used for transformation (-). The individual wt fragments are indicated as well as the size range of the interrupted *dnaJ* genes carrying the *cat* cassette. The interrupted *dnaJ*s (*dnaJ::cat*) were approximately 1000 bp larger than the wt fragments.

analysed growth of the fully segregated *Synechocystis* $\Delta dnaJ$ strains under heat- and cold-stress conditions. Cells were cultivated at normal growth temperature (34 °C) as well as at 42 and 20 °C, and growth of the cultures was followed by measuring OD₇₅₀. In Fig. 4(a–c), growth curves of *Synechocystis* wt and the fully segregated *dnaJ* deletion strains are shown. Under all tested growth conditions, only the $\Delta sll0897$ strain displayed a reduced growth rate compared with the wt and the other $\Delta dnaJ$ strains. While at normal growth temperature this growth defect was not highly pronounced (Fig. 4a), the growth defect was more dramatic under temperature-stress conditions (20 and 42 °C).

Upon cold stress, reduced growth of the $\Delta sll0897$ strain was observed (Fig. 4b), whereas the strain stopped growing completely upon heat stress after approximately 20 h (Fig. 4c). The calculated doubling times of the individual strains are summarized in Fig. 4(d). While the doubling times of most strains were very similar to those of the wt strain under the tested growth conditions, only the doubling time of the $\Delta sll0897$ strain was already increased at 34 °C. At

20 °C, this strain had a doubling time of approximately 90 h, and the doubling time at 42 °C could not be defined, since the strain stopped growing (Fig. 4c).

Cooperativity of DnaJ functions in *Synechocystis*

The results presented above indicate that most of the *Synechocystis* *dnaJs* can be deleted, and thus either the individual DnaJ proteins have specialized functions which are not required under the chosen growth conditions, or individual DnaJs can replace each other (except Sll1933), at least to some extent. The existence of multiple DnaJ proteins in *Synechocystis* raises the question of the extent to which the functions of the individual proteins overlap and how specialized the individual DnaJs are. To answer this question we aimed to delete two *dnaJ* genes in combination and determine whether the functions of the two DnaJs could still be carried out by the remaining representatives. Therefore, all completely segregated *Synechocystis* strains carrying a single *dnaJ* disruption (see above and Fig. 3)

Fig. 4. Growth of the wt and the $\Delta dnaJ$ strains at different temperatures. *Synechocystis* wt and $\Delta dnaJ$ strains were adjusted to OD₇₅₀ 0.05 in BG11 medium. The cultures were grown autotrophically under normal (34 °C) or under heat (42 °C) or cold (20 °C) stress conditions. Growth was monitored by measuring OD₇₅₀. (a) Growth at normal growth temperature (34 °C). Only $\Delta sll0897$ (orange) showed a slightly reduced growth. (b, c) Growth of the $\Delta dnaJ$ strains under (b) cold- (20 °C) and (c) heat-stress conditions (42 °C). The $\Delta sll0897$ strain (orange) stopped growing ~24 h after shifting the cells from 34 to 42 °C. Growth curves: black, wt; red, $\Delta sll0093$; orange, $\Delta sll0897$; yellow, $\Delta sll0909$; green, $\Delta sll1011$; light blue, $\Delta sll1384$; dark blue, $\Delta sll1666$; violet, $\Delta sll1933$. (d) Doubling times of the wt and the *dnaJ* deletion strains under different growth conditions calculated from the curves shown in (a–c). Disruption of *sll0897* led to reduced growth at all tested temperatures. All strains had an increased doubling time under heat- (red bars) and cold-stress conditions (blue bars) compared with 34 °C (white bars). The growth curves shown in (a–c) were determined at least three times; error bars, SD.

were transformed with a plasmid containing another *dnaJ* gene interrupted with a spectinomycin-resistance cassette (*spec*) (compare Fig. 2). After growth of the double-disruption strains on BG11 medium with increasing amounts of spectinomycin, complete segregation of the second deletion was tested by PCR using genomic DNA of the generated strains as a template, as described above for the single disruptions (data not shown). Disruption of two *dnaJ* genes was tested in all possible combinations and the results are summarized in Fig. 5. In one single case two *Synechocystis* *dnaJ* genes were deleted in combination, since *sll1384* was deleted together with *sll0909*. This indicates that the specific functions of the two encoded DnaJ proteins most likely do not widely overlap and can be taken over by the remaining DnaJ proteins. Besides the $\Delta sll1384\Delta sll0909$ double disruption, no other double disruption could be achieved. Thus, while the physiological functions of most DnaJ proteins can be fulfilled by one (or more) of the remaining DnaJs, loss of the functions of two DnaJs can no longer be compensated.

DISCUSSION

Multiple DnaJ proteins in *Synechocystis*

Multiple DnaJ proteins are common in cyanobacteria and several cyanobacterial genomes contain even more *dnaJ* genes than does *Synechocystis*. For example, the genome of the cyanobacterium *Anabaena* sp. PCC 7120 contains 25 genes encoding proteins possessing a DnaJ-like domain, and 16 of these proteins contain J domains with the conserved HPD motif (Kaneko *et al.*, 2001). Within the genome of *Synechocystis* sp. PCC 6803, seven ORFs have been identified that encode proteins containing a typical J domain with the conserved HPD motif, which is essential for stimulating the ATPase activity of a cognate Hsp70 chaperone. The features and functions of this DnaJ protein family have not, however, been defined at all. Why do cyanobacteria typically contain a large number of DnaJ proteins?

Little is known about the specific functions of the individual *Synechocystis* DnaJ proteins. As can be seen in Fig. 1, only the protein encoded by the *sll0897* gene

contains all domains characteristic of a type I DnaJ protein, whereas the other proteins are classified as type II or type III DnaJ proteins. Expression of *sll0897* restored the growth defect of the *E. coli* $\Delta dnaJ$ strain OD259, in which the main *dnaJ* gene is deleted. However, expression of the type II *dnaJ* gene *slr0093* also resulted in growth restoration, which indicates that Slr0093 can carry out the functions of the *E. coli* DnaJ protein as well, at least to some extent. An important function of a DnaJ protein homologous to the *Synechocystis* Slr0093 protein has also been observed in the cyanobacterium *Synechococcus* sp. PCC 7942. Here, the protein has been suggested to be essential, and expression was induced under heat-stress conditions (Sato *et al.*, 2007). However, the suggested predominant function of this *Synechococcus* protein was somewhat unexpected, since only the single type I DnaJ protein appears to be strictly conserved in cyanobacteria (Sato *et al.*, 2007).

In contrast to Slr0093, expression of the second type II protein Sll1933 and of all type III proteins in the *E. coli* $\Delta dnaJ$ strain did not result in complementation of the growth defect. While these data certainly indicate a privileged DnaJ function for Sll0897 and Slr0093, such a conclusion has to be drawn with great caution, since (e.g.) Sll1933 was expressed to an extent different from that of (e.g.) Sll0897, and increased expression of Sll1933 might also result in complementation of the *E. coli* $\Delta dnaJ$ strain growth defect at elevated temperatures. Furthermore, cyanobacterial proteins were analysed in a heterologous host, and the function of DnaJ depends, for example, on specific DnaJ–DnaK interactions. Any specificity is not automatically transferred from one species to the other.

However, while the results presented in Fig. 4 strongly suggest that Sll0897 is the principal Hsp40 protein in *Synechocystis*, the results of the mutational analyses (Fig. 3) indicate that the *sll0897* gene can be disrupted and thus this gene does not appear to be absolutely essential in *Synechocystis*. This is in agreement with observations made in other bacteria, such as *E. coli*. In *E. coli*, the gene encoding the prototypic DnaJ protein can be completely deleted, resulting in a temperature-sensitive growth phenotype (Georgopoulos *et al.*, 1980), as also observed in the *Synechocystis* $\Delta sll0897$ strain. In *Synechocystis*, only disruption of *sll1933* could not be achieved, which suggests a

		cat					
		$\Delta slr0093$	$\Delta sll0897$	$\Delta sll0909$	$\Delta sll1011$	$\Delta sll1384$	$\Delta sll1666$
spec	$\Delta slr0093$		-	-	-	-	-
	$\Delta sll0897$	-		-	-	-	-
	$\Delta sll0909$	-	-		-	+	-
	$\Delta sll1011$	-	-	-		-	-
	$\Delta sll1384$	-	-	+	-		-
	$\Delta sll1666$	-	-	-	-	-	

Fig. 5. Simultaneous disruption of two *dnaJ* genes in *Synechocystis*. *Synechocystis* strains carrying a single *dnaJ* gene disrupted with a chloramphenicol- or spectinomycin-resistance cassette were transformed with a plasmid carrying a second *dnaJ* gene disrupted with the respective resistance cassette. Only *sll0909* and *sll1384* could be disrupted simultaneously.

specific and vital function for this gene in *Synechocystis*. Interestingly, *sll1933* is genetically linked to the *sll1932* gene, which encodes the *Synechocystis* DnaK3 protein (Rupprecht *et al.*, 2007), since both genes are organized in a gene cluster. A deletion analysis of the *Synechocystis* *dnaK* genes has recently indicated that deletion of this gene is also not possible (Rupprecht *et al.*, 2007). In the genomes of 34 completely sequenced cultured cyanobacteria, which are accessible via CyanoBase (Nakamura *et al.*, 2000), conserved gene clusters encoding Sll1932 (DnaK3) and Sll1933 homologues are present. Homologues of Sll1932 (DnaK) and Sll1933 (DnaJ) interact with thylakoid membranes in the cyanobacterium *Synechococcus* sp. PCC 7942, and an involvement in thylakoid membrane biogenesis has been suggested (Nimura *et al.*, 1996; Sato *et al.*, 2007). Thus, Sll1933 appears to be the only essential DnaJ protein in *Synechocystis* and it is likely that Sll1933 interacts functionally with DnaK3. The specific function of this conserved DnaK–DnaJ pair might be linked to thylakoid membrane biogenesis. Such an assumption is further supported by the observation that homologues of Sll1933, as well as of Sll1932, are not encoded in the genome of the cyanobacterium *Gloeobacter violaceus*, the only cyanobacterium known thus far which does not contain internal thylakoid membranes (Nakamura *et al.*, 2003; Rippka *et al.*, 1974).

Three out of the four type III DnaJ proteins contain specific domains in their protein sequences besides the DnaJ domain. The DnaJ protein Sll1011 contains a PRP domain at its C terminus. Such domains are defined as sequences containing tandemly repeating sequences with the consensus [S/T/A/V][D/N][L/F][S/T/R][G] (Vetting *et al.*, 2006). While proteins containing PRP domains can be found in pro- and eukaryotes, cyanobacteria in particular are enriched with proteins containing this domain (Vetting *et al.*, 2006). The function of the domain is, however, largely cryptic and has been analysed in only a few cases. A recent analysis of a Δ *sll1384* *Synechocystis* strain has indicated that this DnaJ protein could be involved in phototaxis (Chen & Xu, 2009). The DnaJ protein Sll1384 contains a TPR motif at its C terminus, and such TPR domains can be found in diverse proteins and are typically involved in protein–protein interactions (Blatch & Lssle, 1999). Interestingly, Hsp70 proteins are often interlinked with the Hsp90 chaperone system in cells, and specific contacts are mediated by co-chaperones containing a TPR motif (Song & Masison, 2005). Thus, it is intriguing to speculate that Sll1384 acts as a connection between *Synechocystis* Hsp70 (DnaK) and Hsp90 proteins, and this will be further investigated in future experiments. The protein Sll1666 contains a transmembrane region following the J domain, and thus this protein is anchored in internal membranes in *Synechocystis*. Transmembrane DnaJ proteins have been described as involved in protein transport across the endoplasmic reticulum and the mitochondrial inner membrane (Dudek *et al.*, 2002; Truscott *et al.*, 2003). Therefore, Sll1666 links the cytoplasmic DnaK system to

internal membranes and membrane-associated cellular functions.

With the exception of *sll1933*, all other *dnaJ* genes could be disrupted individually. This observation indicates either that the majority of DnaJs have specialized functions which are not required under the chosen growth conditions, or that after deletion of a single *dnaJ* gene the physiological functions of the encoded protein are carried out by the remaining DnaJ proteins. In line with this, the single deletion strains did not show a phenotype different from that of the wt strain, with the exception of the Δ *sll0897* strain (see below) (Fig. 4). However, deletion of two *dnaJ* genes in combination was only possible in the case of *sll1384* and *sll0909*, which both encode type III DnaJ proteins, and thus in most cases the specific functions of two DnaJ proteins could no longer be fulfilled by the remaining DnaJ proteins. Therefore, a subset of DnaJs appears to be essential for cell viability in *Synechocystis*. Since only *sll0909* and *sll1384* could be deleted in combination (Fig. 5), the encoded proteins are most likely involved in cellular processes which either are not essential for normal cell growth and viability (under laboratory conditions) and/or can be carried out by the remaining DnaJs. Both proteins are expressed in *Synechocystis* (Wegener *et al.*, 2010), and thus if these two proteins have overlapping functions, the function of (at least) a third DnaJ protein must also overlap to allow complete segregation of the Δ *sll0909* Δ *sll1384* strain. However, the observation that most DnaJ proteins cannot be deleted in combination and thus appear to have specialized functions in *Synechocystis* indicates a highly diverged DnaJ chaperone network in the *Synechocystis* cytoplasm.

Sll0897 is the main, yet dispensable, DnaJ heat-shock protein in *Synechocystis*

While the results shown in Fig. 2 clearly indicate that Sll0897 can actually replace the function of the *E. coli* DnaJ protein, the results of the deletional analysis were somewhat unexpected. In contrast to *sll1933*, disruption of the gene encoding the only type I DnaJ protein was possible in *Synechocystis*. However, disruption of *sll0897* resulted in a phenotype different from that of the wt strain. The Δ *sll0897* strain displayed a reduced growth rate even at a normal growth temperature, whereas growth was highly impaired under cold-stress growth conditions, and under heat-stress conditions the strain was unable to grow. These results suggest that *sll0897* has a predominant and essential function in *Synechocystis*. The temperature-sensitive phenotype observed with the Δ *sll0897* strain is frequently observed after deletion of a *dnaJ* gene encoding a (type I) DnaJ protein, and thus Sll0897 appears to be critically involved in cold- and heat-stress adaptation in *Synechocystis*. Expression of a canonical DnaJ protein is typically induced under cold- or heat-stress growth conditions in other organisms, including *E. coli* (Bardwell *et al.*, 1986). It has to be noted that other DnaJ proteins

might prove to be essential in *Synechocystis* under stress conditions that were not analysed in the present study.

Taken together, based on the domain structure of the protein and on the observed growth defects under cold- and heat-stress conditions, Sll0897 can be defined as the canonical DnaJ heat-shock protein in *Synechocystis*.

Conclusions

In the present study we have analysed multiple *dnaJ* genes of *Synechocystis*. While the two proteins Sll0897 and Sll0093 can complement the growth defect of an *E. coli* Δ *dnaJ* strain, only disruption of *sll0897* results in a growth defect in *Synechocystis* at elevated temperatures. Thus, based on the domain structure and the observed phenotype following disruption, Sll0897 can be classified as the predominant DnaJ protein in *Synechocystis*. However, while most *dnaJ* genes could be individually deleted, deletion of the gene encoding the DnaJ protein Sll1933 was impossible, which suggests an essential, yet undefined, function for Sll1933. All remaining *dnaJ* genes could be deleted without resulting in an altered phenotype, which indicates that the functions of these DnaJ proteins either are not critical under laboratory growth conditions or are carried out by the remaining DnaJs. Nevertheless, only the two *dnaJ* genes *sll0909* and *sll1384* could be disrupted in combination, suggesting physiological functions for the two encoded proteins that can be accomplished by the remaining DnaJs in the double-disruption strain and which most likely are not overlapping.

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