The role of a gene cluster for trehalose metabolism in dehydration tolerance of the filamentous cyanobacterium *Anabaena* sp. PCC 7120

Akiyoshi Higo,^{1,2} Hiroshi Katoh,³ Kazuko Ohmori,⁴ Masahiko Ikeuchi² and Masayuki Ohmori¹

Correspondence

Masayuki Ohmori

ohmori@molbiol.saitama-u.ac.jp

¹Department of Molecular Biology, Faculty of Science, Saitama University, 255 Shimo-Ohkubo, Sakura-ku, Saitama City, Saitama 338-8570, Japan

²Department of Life Sciences (Biology), University of Tokyo, Komaba 3-8-1, Meguro, Tokyo 153-8902, Japan

³Department of Plant Functional Genomics, Life Science Research Center, Mie University, 1577 Kurimamachiya-cho, Tsu City, Mie 514-8507, Japan

⁴Department of Life Sciences, Showa Women's University, 1-7 Taishido, Setagaya, Tokyo 154-8533, Japan

Expression of the genes for trehalose synthesis (*mts* and *mth*, encoding maltooligosyl trehalose synthase and hydrolase) and trehalose hydrolysis (*treH*) in *Anabaena* sp. PCC 7120 was up-regulated markedly upon dehydration. However, the amount of trehalose accumulated during dehydration was small, whereas a large amount of sucrose was accumulated. Northern blotting analysis revealed that these genes were transcribed as an operon. Gene disruption of *mth* resulted in a decrease in the trehalose level and in tolerance during dehydration. In contrast, gene disruption of *treH* resulted in an increase in both the amount of trehalose and tolerance. These results suggest that trehalose is important for the dehydration tolerance of this cyanobacterium. The amount of trehalose accumulated during dehydration was small, corresponding to 0.05–0.1 % of dry weight, suggesting that trehalose did not stabilize proteins and membranes directly during dehydration. To reveal the role of trehalose, the expression profiles of the wild-type strain and gene disruptants during dehydration were compared by using oligomeric DNA microarray. It was found that the expression of two genes, one of which encodes a cofactor of a chaperone DnaK, correlated with trehalose content, suggesting that a chaperone system induced by trehalose is important for the dehydration tolerance of *Anabaena* sp. PCC 7120.

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INTRODUCTION

The N₂-fixing filamentous cyanobacterium *Nostoc commune* and the unicellular cyanobacterium *Chroococcidiopsis* sp. show marked desiccation tolerance (Potts, 1994, 1999). Mechanisms of desiccation tolerance in these organisms have been studied mainly at the structural or biochemical level (Hill *et al.*, 1994; Potts, 1994, 1999; Scherer & Potts, 1989; Shirkey *et al.*, 2003); the molecular mechanism of desiccation tolerance is poorly understood.

The complete genome sequence of the filamentous cyanobacterium *Anabaena* sp. PCC 7120 (hereafter *Anabaena* PCC 7120) has been determined (Kaneko *et al.*, 2001). This cyanobacterium, which is a very close relative of the desiccation-tolerant *Nostoc* sp. HK-01 based on the sequence

of 16S rRNA (Katoh *et al.*, 2003), is also tolerant to dehydration (Katoh *et al.*, 2004). Accordingly, DNA segment microarray analysis was performed to elucidate genome-wide gene expression during dehydration in *Anabaena* PCC 7120. It was revealed that dehydrated *Anabaena* cells exhibit an increase in the expression of many genes, in particular, a marked increase in the expression of those for trehalose metabolism (Katoh *et al.*, 2004).

Trehalose is a non-reducing disaccharide in which two glucose units are linked in an α , α -1,1-glycosidic linkage, and it is widely distributed in various organisms including bacteria, yeasts, fungi, invertebrates and plants (Elbein *et al.*, 2003). Trehalose functions as a source of energy as the main blood sugar in insects (Becker *et al.*, 1996), and as a protectant of proteins and membranes against various stresses such as osmosis, heat and dehydration (Bell *et al.*, 1992; Crowe *et al.*, 1998; Elbein *et al.*, 2003; Giaever *et al.*, 1988; Kandrór *et al.*, 2002). Fungal spores, cysts of the brine

Supplementary tables showing genes up- or down-regulated by dehydration stress are available with the online version of this paper.

shrimp *Artemia* and the desert resurrection plant *Selaginella* accumulate large amounts of trehalose (as much as 20% of their dry weight) under dried-out conditions, which is thought to be essential to the dry stress tolerance of these organisms (Crowe *et al.*, 1998). Recently it has also been suggested that trehalose or its precursor trehalose 6-phosphate acts as a signalling molecule in higher plants (Avonce *et al.*, 2004; Eastmond *et al.*, 2002; Elbein *et al.*, 2003; Paul *et al.*, 2001; Schluepmann *et al.*, 2003; Wingler, 2002).

No reports have yet been published on trehalose accumulation in *Anabaena*. Under salt or osmotic stress, all isolates belonging to the genus *Anabaena* that have been examined accumulate large amounts of sucrose (a non-reducing disaccharide like trehalose), but not trehalose (Hagemann & Marin, 1999; Reed *et al.*, 1986, 1984). In the case of *Nostoc*, a genus related to *Anabaena*, few species accumulate trehalose, whereas most accumulate sucrose as a compatible solute under salt or osmotic shock (Reed *et al.*, 1984). In addition, it was shown that desiccated *Nostoc commune* accumulated trehalose as well as sucrose (Hill *et al.*, 1994). However, no research has been reported on the genes for trehalose synthesis in cyanobacteria.

Some prokaryotes such as *Sulfolobus* (Nakada *et al.*, 1996a, b) and *Arthrobacter* (Maruta *et al.*, 1995) have a trehalose synthesis pathway, namely maltooligosyl trehalose synthase (Mts) and maltooligosyl trehalose hydrolase (Mth). Mts converts the reducing terminal α -1,4-linked residue of glucose polymers such as glycogen and maltooligosaccharide to α -1,1 linkage, while Mth subsequently produces trehalose.

In the genomic sequence of *Anabaena* PCC 7120, *all0168*, *all0167* encoding Mth and Mts respectively, and *all0166*

encoding trehalase, which are considered to be genes for trehalose-metabolizing enzymes, constitute a gene cluster (Fig. 1), and all of them are up-regulated by dehydration stress (Katoh *et al.*, 2004). A survey of genome sequences revealed that the gene cluster consisting of *mth*, *mts* and *treH* is characteristic of *Anabaena* PCC 7120 and related species such as *Anabaena variabilis* and *Nostoc punctiforme*. Therefore, a crucial role of trehalose-metabolizing enzymes in dehydration tolerance was expected. In this research, we investigated the role of trehalose or the genes for trehalose-metabolizing enzymes *mth*, *mts* and *treH* in dehydration tolerance in *Anabaena* PCC 7120. It was found that trehalose, accumulated only in small quantities, may be important for the dehydration tolerance of *Anabaena* PCC 7120 by regulating the expression of genes related to a molecular chaperone.

METHODS

Strains, growth conditions, and dehydration stress treatments. *Anabaena* PCC 7120 (wild-type strain) and gene disruptant mutants were grown at 30 °C at 40 $\mu\text{E m}^{-2} \text{s}^{-1}$ in nitrogen-free modified Detmer's medium (MDM₀) (Watanabe, 1960) until late-exponential phase. Liquid culture was bubbled with air containing 1.0% (v/v) CO₂.

Desiccation stress was imposed on *Anabaena* cells as described in our previous report (Katoh *et al.*, 2004). A 30 ml portion of cell culture was filtered on cellulose acetate filter paper (0.45 μm pore size, 47 mm diameter; Advantec) and dried for the indicated time at 30 °C at 30–40 $\mu\text{E m}^{-2} \text{s}^{-1}$ in a Petri dish.

Northern blotting analysis. RNA was extracted from the *Anabaena* cells as described previously (Katoh *et al.*, 2004). Northern blotting analysis was carried out with DIG-labelled DNA probes. Internal

Fig. 1. Schematic representation of genes for trehalose-metabolizing enzymes in *Anabaena* PCC 7120 (upper figure) and reactions catalysed by their products (lower figure).

fragments of *mtH*, *mts* and *treH* were amplified by PCR and labelled with a DIG PCR synthesis kit (Roche Molecular Biochemicals) using primers as previously reported (Katoh *et al.*, 2004).

Ten micrograms of RNA was separated on formaldehyde agarose gels and transferred to a Hybond-N⁺ membrane (Amersham Biosciences) by vacuum blotting. Pre-hybridization, hybridization with DIG-labelled PCR probes and detection with a CSPD chemiluminescence system were carried out according to the user's guide (Roche Molecular Biochemicals).

Inactivation of *all0168*, *all0167* and *all0166*. To inactivate the gene *mtH*, a 1.0 kb DNA fragment from the *mtH* region of *Anabaena* PCC 7120 was amplified by PCR using primers 5'-CTCGAGA-ATTGGCGCTCACTACTT-3' and 5'-GGATCCGGTGAAAGTCA-TCACTCC-3' or 5'-GGATCCCGATCGCCAGGTATTA-3' and 5'-CTCGAGAACCATGTGTACTACCAG-3'. Their respective PCR products were cloned in vector pGEM-T Easy digested with *HincII*. Both plasmids were digested with *Bam*HI and *Pst*I, and ligated with each other, rendering plasmid pTRM11 with a new *Bam*HI restriction site at the centre of *mtH*. The Sp^r-Sm^r cassette excised with *Bam*HI was inserted into the *Bam*HI site present in the inserted DNA fragment of *mtH*, generating plasmid pTRM11-O. The *Xho*I fragment from pTRM11-O was ligated to the vector pRL271 (Cai & Wolk, 1990) digested with *Xho*I, rendering plasmid pTRM11-S.

To inactivate the gene *mts*, a 1.2 kb DNA fragment from the *mts* region of *Anabaena* PCC 7120 was amplified by PCR using primers 5'-GGCTCGAGGAGAAATTTCAAACATTT-3' and 5'-AACTCGAG-GGGAATTTTCAGATAACAC-3'. PCR products were cloned in vector pGEM-T Easy digested with *HincII* to create plasmid pTRM21. The cassette excised with *Hind*III was inserted into the *Hind*III site present in the inserted DNA fragment of *mts*, generating plasmid pTRM21-O. The *Xho*I fragment from pTRM21-O was ligated to the vector pRL271 (Cai & Wolk, 1990) digested with *Xho*I, rendering plasmid pTRM21-S.

To inactivate the gene *treH*, a 1.1 kb DNA fragment from the *treH* region of *Anabaena* PCC 7120 was amplified by PCR using primers 5'-CTCGAGCCGTAGCCCTATTAATTG-3' and 5'-CTCGAGTAAT-AGAATGCGGGGTAG-3'. PCR products were cloned in vector pGEM-T Easy digested with *HincII* to create plasmid pTRM31. The cassette excised with *Xba*I was inserted into the *Xba*I site present in the inserted DNA fragment of *treH*, generating plasmid pTRM31-O. The *Xho*I fragment from pTRM31-O was ligated to the vector pRL271 (Cai & Wolk, 1990) digested with *Xho*I, rendering plasmid pTRM31-S.

mtH, *mts* and *treH* were inactivated by a *sacB*-mediated positive selection for double recombination (Cai & Wolk, 1990). Transformation of *Anabaena* PCC 7120 was performed by a conjugation method (Elhai & Wolk, 1988), and both single and double recombinations were confirmed by PCR or Southern hybridization.

Determination of trehalose and sucrose contents. The low-molecular-mass compounds of dehydrated cells were extracted with 15 ml 80% ethanol for 3 h at 65 °C. After centrifugation, each pellet was washed with 5 ml 80% ethanol and centrifuged again. Both supernatants were combined and vacuum-dried. The residue was redissolved in 0.5 ml water.

After centrifugation for 5 min, the amount of trehalose was determined using trehalase (Sigma). The supernatant (100 µl) was added to the reaction mixtures (100 µl) containing 100 mM MES/KOH (pH 6.0) and 5×10^{-3} U ml⁻¹ trehalase (Sigma), and the liberated glucose was measured using a glucose test kit (Wako). The amount of sucrose was determined by adding 40 µl supernatant to the mixtures (60 µl) containing 50 mM sodium acetate (pH 4.5) and 1 U invertase (Sigma), and the liberated glucose was measured. The pre-existing glucose in each sample was determined in a control reaction lacking

trehalase or invertase and subtracted from the total glucose. The absorbance of chlorophyll extracted by methanol was measured at 665 nm and the chlorophyll concentration calculated from the equation 1 A₆₆₅ unit = 13.42 µg chlorophyll ml⁻¹ (Mackinney, 1941).

Enzyme assays. Cells on filter paper were suspended in a disruption buffer [20 mM HEPES/NaOH (pH 7.0), 5 mM 6-amino-n-caproic acid, 1 mM benzamidine.HCl]. Cells were broken using a bead beater (Biospec Products) in the presence of 1 ml zircon beads. Cell debris was removed by centrifugation at 10 000 g for 5 min. The supernatant was centrifuged at 100 000 g for 30 min, and the pellet was discarded. The supernatant was desalted in an NAP-10 column (Amersham) equilibrated with the same buffer. The resulting supernatants, referred to as crude cell extracts, were used in enzyme assays.

The reactions were sustained from 0 to 40 min at 30 °C and stopped by boiling the reaction mixtures for 5 min. Trehalose synthesis activity was measured by incubating cell extracts in 200 µl of 20 mM HEPES/NaOH buffer (pH 7.0) containing 1% maltoheptaose, and the trehalose formed was determined using trehalase (Sigma). Trehalase activity was measured by incubating cell extracts in 200 µl 20 mM HEPES/NaOH buffer (pH 7.0) containing 20 mM trehalose. The boiled mixtures were centrifuged for 5 min, and the glucose formed in the supernatant was determined by a glucose test kit (Wako).

Dehydration tolerance assay. Cells on filter paper were suspended in MDM₀. The suspended cultures were diluted with MDM₀, and 10 µl samples of cell cultures were spotted on MDM₀ agarose plates, followed by incubation for 3 days at 30 °C at 30 µE m⁻² s⁻¹. Viability was calculated as the numbers of colonies formed.

Preparation of fluorescent cDNA. A single-stranded fluorescent cDNA probe was prepared using SuperScript II (Invitrogen) with 5 µg total RNA. First, a mixture of RNA and *Anabaena* primer mix (Sigma) in a 9 µl volume was heated to 90 °C for 5 min, and then gradually cooled to 42 °C for 20 min. Reverse transcription was performed at 42 °C for 90 min in a 20 µl volume containing 5 µg RNA, *Anabaena* primer mix (3.44 nM each ORF), 1 × first-strand buffer (50 mM Tris/HCl, 75 mM KCl, 3 mM MgCl₂, pH 8.3), 10 mM DTT, 500 µM each of dATP, dGTP, dCTP, 200 µM dTTP, 100 µM Cy3-dUTP or Cy5-dUTP, 200 U SuperScript II Reverse Transcriptase and 20 U RNase inhibitor (Takara). RNA in the reaction mixtures was hydrolysed by incubating at 70 °C after adding 10 µl 0.1 M NaOH, after which the mixtures were neutralized by adding 15 µl 1 M Tris/HCl (pH 7.5). Two reaction mixtures (one labelled with Cy3 and another labelled with Cy5) were combined and purified by a QIAquick PCR purification kit (Qiagen) to remove unincorporated fluorescent nucleotides. Ten microlitres of 20 × SSC was added to a cDNA solution (29 µl), followed by denaturation at 95 °C for 5 min. The mixture was cooled to room temperature, then 2 µl 10% SDS was added.

DNA microarray analysis. The *Anabaena* oligonucleotide microarray (Sigma) covers more than 99% (5336/5368) of the annotated *Anabaena* PCC 7120 genes on the main chromosome. Some genes for transposase and photosynthesis D1 were not included in the microarray to prevent cross-hybridization. Primers for synthesis of fluorescent cDNA (hereafter referred to as *Anabaena* primer mix) and the oligonucleotide probes of 5336 respective genes were designed and synthesized by Sigma. Each slide glass was coated, printed with 5472 features including 5336 54–70-mer oligonucleotides and controls, and prehybridized by Asahi Techno Glass.

DNA microarray analysis was mainly performed as described by Ehira *et al.* (2003). The relative expression ratio for each oligo DNA spot was defined as the normalized fluorescence intensity for each DNA spot at each time point relative to that at zero time. The experiment was carried out twice independently with different combinations of Cy

dyes. Thus, the relative ratio for each DNA spot is represented by four measurements. Clustering analysis was done by J-Express (Dysvik & Jonassen, 2001).

Real-time PCR assay. Template cDNA was prepared using SuperScript II (Invitrogen) with 1 µg total RNA. First, a mixture of RNA, Anabaena primer mix (Sigma) and 16S rRNA reverse primer in an 11 µl volume was heated to 90 °C for 5 min, and then gradually cooled to 42 °C for 20 min. The reverse transcription was performed at 42 °C for 90 min in a 20 µl volume containing 1 µg RNA, Anabaena primer mix (0.86 nM each ORF), 50 nM 16S rRNA reverse primer, 1 × First strand buffer (50 mM Tris/HCl, 75 mM KCl, 3 mM MgCl₂, pH 8.3), 10 mM DTT, 500 µM each of dATP, dTTP, dGTP, dCTP, 200 U SuperScript II Reverse Transcriptase and 20 U RNase inhibitor (Takara). The cDNA synthesis reaction mixture was diluted fivefold before being used for PCR.

The subsequent PCR was performed in a total volume of 20 µl containing 1 µl cDNA, 0.25 µM of each of the primers and 10 µl 2 × SYBR Premix Ex Taq (Takara). Amplification and detection of DNA were performed with a DNA Engine Opticon 2 (MJ Research). The initial denaturing time was 10 s, followed by 40 PCR cycles consisting of 95 °C for 5 s and 60 °C for 30 s. A melting curve was obtained after the PCR cycles. Quantification was performed using Opticon Monitor Analysis software 2.02 (MJ Research).

Primers for the PCR reactions were designed to give a PCR product between 100 and 250 bp. The genes tested for expression levels by real-time PCR were as follows: *all0641*, forward primer 5'-GCCCAATTG-ATATTGCTCCA-3' and reverse primer 5'-TGTAGCGGGAGAATCT-GTTG-3'; *alr2445*, forward primer 5'-TAACTGAGCCAGTGGCAA-TC-3' and reverse primer 5'-TGCCTAC-TGCGTTCACTAAC-3'. The internal control gene was 16S rRNA: forward primer 5'-TGTAG-CGGTGAAATGCGTAG-3' and reverse primer 5'-TTCACACTGCGTGCCTACT-3'.

RESULTS

Gene cluster for trehalose metabolism in *Anabaena* PCC 7120

The gene products of *all0167* and *all0168* show 38 % and 45 % identity to Mts and Mth from *Sulfolobus acidocaldarius*, respectively, which produce trehalose from the reducing end

of α-1,4-glucan by sequential reactions (Maruta *et al.*, 1996). The gene product of *all0166* shows 32 % identity to trehalase from *Saccharomyces cerevisiae*, which hydrolyses trehalose (Kopp *et al.*, 1993). Thus, genes *all0168*, *all0167* and *all0166* were named *mth*, *mts* and *treH*, respectively.

A transcript of approximately 6.6 kb was detected by Northern blotting analysis with probes of *mth*, *mts* or *treH* in RNA from the *Anabaena* PCC 7120 cells dehydrated for 30 min (Fig. 2). Since the length from the beginning of *mth* to the end of *treH* is approximately 6.2 kb, this result showed that these three genes constituted an operon.

To elucidate the function of this operon, the *mth* gene at the top of the operon was inactivated by insertion of an Sp^r-Sm^r cassette, and the resultant mutant strain was named TRM11. In this disruptant, all trehalose-metabolizing enzymes in the operon were predicted to be inactive, since the cassette contains potent transcriptional terminators. Insertional mutants of *mts* and *treH* (designated TRM21 and TRM31, respectively) were also constructed. Using the wild-type and these mutant strains, the enzyme activities of trehalose synthesis (Mts + Mth) and trehalose hydrolysis (trehalase) during dehydration stress were determined with crude cell extracts.

Under liquid culture conditions, the activity of neither Mts + Mth nor trehalase was detected in the wild-type strain or in those three gene disruptants. On the other hand, Mts + Mth activity of about 1 nmol trehalose min⁻¹ (mg protein)⁻¹ was detected in the wild-type strain under dehydration conditions (dehydrated for 3–12 h). The trehalase synthesis activity in strain TRM31 (*treH*::Ω) was about the same as that in the wild-type strain, whereas no activity was detected in strains TRM11 (*mth*::Ω) or TRM21 (*mts*::Ω). These results show that Mts + Mth of *Anabaena* PCC 7120 are active during dehydration. Trehalase activity of about 2–3 nmol glucose min⁻¹ (mg protein)⁻¹ was detected in the wild-type strain, whereas no trehalase activity was detected throughout dehydration

Fig. 2. The genes for trehalose synthesis (*mth*, *mts*) and trehalose hydrolysis (*treH*) constitute an operon. RNA was extracted from *Anabaena* PCC 7120 cells before (lane C, control) and after (lane D) dehydration for 30 min, and probed with DIG-labelled DNA fragments of *mth* (a), *mts* (b) or *treH* (c). The 6.6 kb transcript is arrowed.

stress in strain TRM31. In addition, no trehalase activity was detected in strains TRM11 or TRM21 under dehydration conditions; this indicates polar effects on *treH* expression, suggesting that the genes for trehalose metabolism are transcribed as an operon.

Accumulation of sugars during dehydration stress

The change in the wet weight of *Anabaena* PCC 7120 during dehydration was determined. The wet weight was previously shown to decrease linearly during the first 9 h of dehydration, and to remain constant thereafter at least up to 24 h (Kato *et al.*, 2004). The same results were obtained with the gene disruptants (data not shown). We thus designated the initial 9 h of dehydration as the early dehydration phase, and after 9 h as the late dehydration phase.

Fig. 3(a) shows that the trehalose content of the wild-type strain gradually increased during dehydration, although the amount was small (corresponding to 0.05–0.1 % of dry weight). The amount of trehalose in strains TRM11 and TRM31 was similar to that of the wild-type strain in its early dehydration phase. However, at 9 and 24 h of dehydration, the trehalose content was lower in strain TRM11 and higher in strain TRM31 than in the wild-type strain. After dehydration for 24 h, the trehalose amount of strain TRM11 was lower and that of TRM31 was higher than that of the wild-type strain.

Other sugar molecules accumulated during dehydration in *Anabaena* PCC 7120 were determined by HPLC; this showed accumulation of large amounts of sucrose (data not shown), so we measured sucrose contents during dehydration precisely by an invertase assay (Fig. 3b). The sucrose content increased linearly during the early dehydration phase, remaining roughly constant during the following phase. The accumulation pattern of sucrose in strains TRM11 and TRM31 was similar to that in the wild-type strain, although the sucrose amount of the gene disruptants was about 60 % of that in the wild-type strain after 24 h of dehydration.

Dehydration tolerance

To determine the physiological importance of trehalose and sucrose during dehydration, tolerance during dehydration stress of wild-type and gene disruptants was compared. We counted numbers of colonies after spotting resuspended culture of dehydrated cells on agar plate. Fig. 3(c) shows that the colony-forming ability of the wild-type gradually decreased with dehydration time, while that of strains TRM11 and TRM21 decreased more rapidly after 9 h of dehydration. In contrast to strains TRM11 and TRM21, the colony-forming ability of strain TRM31 was slightly higher than that of the wild-type strain. Fig. 3(a, c) shows that the strain with a greater accumulation of trehalose has a higher dehydration tolerance at the late dehydration phase.

Fig. 3. (a, b) Accumulation of trehalose (a) and sucrose (b) during dehydration stress. After drying for the indicated time, low-molecular-mass compounds were extracted as described in Methods. Trehalose or sucrose was measured using trehalase or invertase (Sigma), respectively. The experiment was carried out more than three times with independently grown cultures; means $\pm$ SD are shown. Chl, chlorophyll. (c) Changes in colony-forming ability during dehydration stress. Cells were dried, and then resuspended in growing medium. Cell culture was then spotted on MDM₀ solid medium, and after 3 days of incubation at 30 °C, numbers of colonies were counted. The dehydration tolerance assay was repeated twice and each was done in duplicate; means $\pm$ SD are shown. ●, *Anabaena* PCC 7120; ▲, TRM11 (*mth*::Ω); ○, TRM21 (*mts*::Ω); ■, TRM31 (*treH*::Ω).

Gene expression and trehalose content

We compared the whole gene expression profiles of strains PCC 7120, TRM11 and TRM31 during the dehydration stress by oligo DNA microarray analysis. The expression profiles of strains PCC 7120, TRM11 and TRM31 were the same before dehydration treatment (data not shown). The cells were then subjected to 0, 3 and 9 h of dehydration stress, and the relative ratio of gene expression was calculated by comparing the value at zero time with that at each time-point. Many genes were up- or down-regulated by dehydration stress in each strain; a list of such genes in the wild-type strain is shown in Tables S1 and S2, available as supplementary data with the online version of this paper. It was found that *mth* and *mts* were up-regulated transiently in early dehydration phase and that at 9 h of dehydration, genes of heat-shock proteins in particular were up-regulated. Many other genes, including *spsA*, encoding sucrose-6-phosphate synthase (Cumino *et al.*, 2002), were up-regulated

constantly during dehydration. Many genes related to photosynthesis and ribosomal protein were down-regulated in the early dehydration phase, whereas genes for nitrogen fixation and photosynthesis I were down-regulated in the late dehydration phase.

It was noted from the microarray data and cluster analysis using J-Express software (Dysvik & Jonassen, 2001) that the expression levels of only two genes, *alr2445*, encoding GrpE (DnaK cofactor), and *all0641*, encoding a hypothetical protein, were correlated with trehalose content. Expression levels of no other genes were correlated with trehalose content. At 3 h of dehydration, the relative ratio of these two genes did not differ much among the three strains (wild-type, TRM11 and TRM31). At 9 h of dehydration, when trehalose contents differed among the three strains, the relative ratio of up-regulation in *alr2445* was 2.5-fold lower in strain TRM11 but 1.3-fold higher in TRM31 than that in the wild-type strain (Fig. 4a). The relative ratio of up-regulation in *all0641* was 1.6-fold lower in strain TRM11 but 1.9-fold higher in TRM31 than that in the wild-type strain at 9 h of dehydration (Fig. 4b). The relative ratios of up-regulation in these two genes were parallel to the trehalose content. We then performed real-time quantitative PCR on those two genes (Fig. 4c, d). The results were consistent with those of DNA microarray analysis. It was concluded

that the expression level of the two genes is correlated with the trehalose contents.

DISCUSSION

Although expression of genes for trehalose synthesis, *mth* and *mts*, as well as that for trehalose degradation, *treH*, exhibited marked increase upon dehydration (Katoh *et al.*, 2004), trehalose did not accumulate so much (Fig. 3a). So far, many reports have revealed that trehalose is rapidly accumulated in large quantities under such stresses as salt, osmosis, drought, heat and cold by Mts+Mth or/and trehalose-6-phosphate synthase/phosphatase (TPS/TPP) (Elbein *et al.*, 2003; Wolf *et al.*, 2003). TPS catalyses the transfer of glucose from UDP-glucose to glucose 6-phosphate to produce trehalose 6-phosphate and UDP, and TPP catalyses the dephosphorylation of trehalose 6-phosphate to trehalose. The low level of accumulation of trehalose may be attributed to the unique gene structure for trehalose metabolism (Fig. 1). The genes *mth*, *mts* and *treH* constitute an operon in *Anabaena* PCC 7120 (Fig. 2). In addition, trehalase activity was not detected in strain TRM11 (*mth::Ω*), which supported the view that the expression of *mth*, *mts*, and *treH* is under the same regulation. This cluster of genes is conserved among the family

Fig. 4. Correlation of expression of two genes, *alr2445* (a, c) and *all0641* (b, d), with trehalose content during dehydration. (a, b) Oligo DNA microarray analysis of *alr2445* (a) and *all0641* (b) during dehydration was performed in strains PCC 7120 (black bars), TRM11 (grey bars) and TRM31 (white bars). Data are represented as the ratio of normalized intensity of dehydrated cells to that of non-dehydrated cells. (c, d) Real-time PCR analysis of *alr2445* (c) and *all0641* (d) was performed to confirm the data from DNA microarray analysis. Means ($\pm$ SD in a and b) are shown.

Nostocaceae, suggesting that their coexistence is important for these filamentous cyanobacteria.

In contrast to our finding of a gene cluster in *Anabaena* PCC 7120, genes for trehalose synthesis and degradation in prokaryotes are generally localized separately on the genome, while a pair of genes for trehalose synthesis such as *tps* and *tpp* or *mts* and *mth* are often tightly linked to each other. As an exception, the genes *tps*, *tpp* and *treS* are located tandemly in *Thermus thermophilus* RQ-1, although *treS*, which is involved in trehalose degradation, is assumed to be regulated separately from *tps* and *tpp* (Silva *et al.*, 2003). It has been reported that, although *Escherichia coli* and *S. cerevisiae* accumulate a large amount of trehalose under osmotic or heat stress, a trehalase gene in addition to *tps* and *tpp* was induced under those stresses (Cansado *et al.*, 1998; Horlacher *et al.*, 1996; Zähringer *et al.*, 2000). However, genes for trehalose synthesis and degradation are located separately in both *E. coli* and *S. cerevisiae*, suggesting that they are regulated independently in those organisms.

Considering that Mts, Mth and trehalase catalyse sequential reactions, that is synthesizing trehalose from glycogen and hydrolysing it to glucose, it was postulated that the gene cluster for trehalose metabolism in *Anabaena* PCC 7120 is not for trehalose accumulation, but rather for synthesis of a certain molecule derived from trehalose. Disruption of both *mth* and *treH* resulted in a decrease in the amount of sucrose during dehydration stress (Fig. 3b). However, the increase in the amount of trehalose by disruption of *treH* was about one-tenth the size of the decrease in the amount of sucrose by disruption of *mth* or *treH*, suggesting that sucrose is not synthesized via the trehalose synthesis and hydrolysis pathway. Disruption of *mth* or *treH* might have affected sucrose metabolism indirectly, resulting in a decrease of the sucrose content in strains TRM11 and TRM31.

Trehalose might be metabolized by an enzyme other than trehalase. Accumulation of trehalose in strain TRM11 lacking Mts + Mth activity suggests the possibility that trehalose might be metabolized by another enzyme. The candidate gene for this would be *all1058*, which encodes maltose or trehalose phosphorylase (Katoh *et al.*, 2004). Alternatively, trehalose might be used for the synthesis of exopolysaccharide or lipopolysaccharide, directly or indirectly. Another possible explanation for the low trehalose accumulation is that the enzyme activity of trehalose synthesis is very low *in vivo*. This possibility is supported by the fact that strain TRM31, which retains normal trehalose synthesis activity, did not accumulate much trehalose.

The results in Fig. 3(a, c) show that the strains that accumulated more trehalose exhibited a higher dehydration tolerance. On the other hand, their sucrose contents did not correlate with the dehydration tolerance (Fig. 3b, c). These results suggest that, although the amount of trehalose was small, it is more important than is sucrose for the tolerance of *Anabaena* to the stress. It must be mentioned that the accumulation of trehalose occurred gradually (Fig. 3a),

although induction of the gene cluster for trehalose metabolism was rapid upon dehydration (Katoh *et al.*, 2004). A marked decrease in the tolerance of strain TRM11 was observed after 12 h of dehydration, when water content has decreased and remains constant (Katoh *et al.*, 2004).

Yeast, fungal spores and *Artemia* accumulate trehalose in large quantities under dry stress, and similarly, plants accumulate sucrose in large quantities under dry conditions. These two disaccharides are considered to confer a high dry stress tolerance to those organisms by protecting their proteins and membranes (Crowe *et al.*, 1998). Two hypotheses have been proposed to explain the protective effect of trehalose and sucrose: (1) water replacement and (2) vitrification (Crowe *et al.*, 1998; Potts, 2001). In either case, very large amounts of trehalose or sucrose are required to protect proteins and membranes, at least *in vitro* (Crowe *et al.*, 1998; Potts, 2001). Sano *et al.* (1999) reported that yeast cells require 2–3 % trehalose on a dry weight basis to stabilize proteins and membranes under dehydration *in vivo*. Therefore, sucrose that accumulated in large quantities under dehydration stress, corresponding to 1–2 % of dry weight, might protect the large biomacromolecules in *Anabaena* PCC 7120, whereas trehalose might not.

In addition to stabilization by either water replacement or vitrification, trehalose and sucrose increase the stability of native proteins and assist in the refolding of unfolded polypeptides (Hottiger *et al.*, 1994; Singer & Lindquist, 1998); thus they are referred to as ‘chemical’ chaperones. Since dehydration stress possibly causes an aggregation of proteins (Goyal *et al.*, 2005; Potts, 1994), a chaperone system would be important in protecting the structure of proteins against denaturation and aggregation under dehydration. Large amounts of the disaccharides are required to function as a chemical chaperone (Hottiger *et al.*, 1994). In the present study, a small amount of trehalose and large amounts of sucrose were detected in *Anabaena* cells (Fig. 3a, b), indicating that not trehalose but rather sucrose would be more likely to function as a chemical chaperone.

Some genes, such as *clpB*, related to a ‘molecular’ chaperone were up-regulated in the late dehydration phase (see Supplementary Tables S1 and S2). Furthermore, DNA microarray analysis revealed that there were two possible molecular-chaperone-related genes whose expression levels were correlated with trehalose amounts (Fig. 4). One is *alr2445*, encoding GrpE, and the other is *all0641*, encoding a hypothetical protein which has a DUF1232 Pfam domain (www.sanger.ac.uk/Software/Pfam/) conserved in bacteria, archaea and higher eukaryotes. GrpE is a cofactor of DnaK, a chaperone protein that participates in protein folding (Goloubinoff *et al.*, 2001). In *Synechocystis* sp. PCC 6803, *slr0846*, an orthologue gene of *all0641*, is up-regulated under various stress conditions (Hihara *et al.*, 2003; Inaba *et al.*, 2003; Kanesaki *et al.*, 2002; Paithoonrangarid *et al.*, 2004) and its expression pattern is very similar to those of *slr0170*, encoding DnaK, and *slr1641*, encoding ClpB. ClpB collaborates with a DnaK chaperone system to dissolve and

reactivate aggregated proteins (Shorter & Lindquist, 2005). Taking these observations together, *alr2445* and *all0641* are probably involved in that chaperone system.

The DnaK chaperone system of *E. coli* is inhibited by a high concentration (0.5 M) of trehalose (Diamant *et al.*, 2001; Singer & Lindquist, 1998), and that of *Anabaena* PCC 7120 might be also blocked by a large amount of trehalose. Depending on its compatibility with a molecular chaperone, sucrose but not trehalose might be accumulated in large quantities in *Anabaena* PCC 7120. In conclusion, instead of acting as a chemical chaperone, trehalose would appear to regulate expressions of molecular-chaperone-related genes and be important for *Anabaena* PCC 7120 in acquiring desiccation tolerance. There is an example of the involvement of trehalose in the regulation of chaperone genes. In *S. cerevisiae*, it was suggested that trehalose is involved in the activity of heat-shock transcription factor (Bulman & Nelson, 2005), although the corresponding gene was not found in *Anabaena* PCC 7120. Whether the expression of *alr2445* and *all0641* is regulated directly or indirectly by trehalose in *Anabaena* PCC 7120 is a matter to be tested in future.

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