

## Full Paper

# Water-stress induced trehalose accumulation and control of trehalase in the cyanobacterium *Nostoc punctiforme* IAM M-15

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**We investigated the biochemical properties of the enzymes involved in trehalose metabolism in the cyanobacterium *Nostoc punctiforme* strain IAM M-15 to elucidate the mechanism of trehalose accumulation in response to desiccation and salt stress. There was no detectable trehalose in fully hydrated *N. punctiforme* cells; however, these cells accumulated trehalose upon desiccation. Moreover, NaCl treatment also induced trehalose accumulation. The three genes for trehalose metabolism, *treZ* (maltooligosyltrehalose trehalohydrolase, Mth), *treY* (maltooligosyltrehalose synthase, Mts), and *treH* (trehalase), were found as a gene cluster, and the mRNAs for these genes were detectable at similar levels during desiccation. Trehalase of *N. punctiforme* was heterologously expressed in *E. coli* cells in an active form with a molecular mass of 52 kDa. Trehalase activity was strongly inhibited in the presence of 10 mM NaCl while trehalose synthesis activity remained active in the presence of salt. These data suggest that the rate of trehalose production exceeds that of trehalose hydrolysis under water-stress conditions characterized by increased cellular solute concentrations. In the proposed mechanism, control of trehalase plays an important role in trehalose accumulation in terrestrial cyanobacteria under conditions of extreme desiccation.**

**Key Words**—compatible solute; desiccation tolerance; post-translational regulation; terrestrial cyanobacterium

## Introduction

Desiccated organisms have little to no metabolic activity and rapidly resume metabolism upon rehydration. This phenomenon is termed “anhydrobiosis” (Clegg, 2001; Crowe, 2002; Crowe et al., 1998; Potts, 2001). The desiccated terrestrial cyanobacterium *Nostoc commune* sustains the capacity for cell growth for

over 100 years (Cameron, 1962; Lipman, 1941); thus, *N. commune* is considered an anhydrobiotic microorganism with oxygenic photosynthetic capabilities. Since *N. commune* does not differentiate into akinetes (spores), the mechanisms of its extreme desiccation tolerance most likely involve multiple processes (Potts, 1999, 2001). In natural habits, *N. commune* forms visible colonies that consist of biochemically complex extracellular matrices (Morsy et al., 2008; Wright et al., 2005) and cellular filaments embedded within extracellular polysaccharides (EPSs; Potts, 2000). We previously demonstrated that *N. commune* EPSs play a crucial role in desiccation tolerance, and photosynthetic activity is damaged significantly by desiccation when EPSs are removed (Tamaru et al., 2005).

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The accumulation of trehalose is involved in responses to abiotic stress (Burg and Ferraris, 2008; El-bein et al., 2003; Sakamoto et al., 2009). Trehalose ( $\alpha$ -D-glucopyranosyl-[1, 1]- $\alpha$ -D-glucopyranoside) is a non-reducing disaccharide that is synthesized from  $\alpha$ (1, 4)-linked glucose polymers in *Nostoc* by the TreY-TreZ pathway. Maltooligosyltrehalose synthase (Mts) encoded by the *treY* gene converts the terminal  $\alpha$ (1, 4)-linked residue of the glucose polymer to an  $\alpha$ (1, 1) linkage, and maltooligosyltrehalose trehalohydrolase (Mth) encoded by the *treZ* gene produces free trehalose via cleavage of the terminal disaccharide. In the genomic sequence of *Nostoc* sp. PCC 7120 (the same strain as *Anabaena* sp. PCC 7120), *all0168*, *all0167*, and *all0166* encoding Mth, Mts, and trehalase, respectively, constitute a gene cluster and are simultaneously up-regulated by desiccation stress (Higo et al., 2006; Katoh et al., 2004). Since transcriptional up-regulation of the gene cluster for trehalose synthesis and trehalose hydrolysis cannot fully explain trehalose accumulation in response to desiccation, post-transcriptional regulation most likely controls the cellular level of trehalose in cyanobacteria. Here we investigated changes of trehalose levels in response to matric water stress (desiccation) and osmotic water stress (high salt concentration) and the biochemical properties of the enzymes involved in trehalose metabolism in the cyanobacterium *N. punctiforme* strain IAM M-15, which is closely related strain to *N. commune*.

## Materials and Methods

**Organisms.** The cyanobacterium *Nostoc punctiforme* strain IAM M-15 was obtained from the Institute of Molecular and Cellular Biosciences, University of Tokyo, and grown photoautotrophically in aerated BG11 liquid medium (Rippka, 1988) buffered with 20 mM HEPES-NaOH (pH 7.5) under continuous illumination at 30°C. The cells were harvested at the mid- to late-exponential growth phase.

**Desiccation treatment.** Cells of *N. punctiforme* were harvested by centrifugation and blotted on nylon mesh (9 cm  $\times$  9 cm, 50  $\mu$ m pore size). The cells on the nylon mesh were air-dried under ambient conditions (approximately 25°C and 50 to 80% relative humidity). After measurement of the fresh weight (FW) of the desiccating cells, trehalose and sucrose levels in the cells were determined as described below. The weight of the completely dried cells after 72 h of desiccation was

defined as the dry weight (DW), and the water content was calculated accordingly: (water content) = (FW – DW) / DW.

**Salt-stress treatment.** Cells of *N. punctiforme* were harvested by centrifugation and suspended in 25 mM HEPES-NaOH (pH 7.5) containing 0 to 1 M NaCl. After incubation at room temperature under ambient light conditions, cells were collected by centrifugation, and trehalose and sucrose levels were determined as described below.

**Determination of trehalose and sucrose.** Trehalose and sucrose were determined by gas-liquid chromatography (GLC) essentially as described by Oda et al. (1997). After desiccation or salt stress treatments, *N. punctiforme* cells (20 mg) were frozen in liquid N<sub>2</sub> and ground with a mortar and pestle. After lyophilization, the dry powder of *N. punctiforme* cells was homogenized with 2.5 ml of 80% (v/v) ethanol and heated at 80°C for 10 min. The cells were then centrifuged at 21,500  $\times$  g for 10 min, and 1 ml of the supernatant was dried using a centrifugal concentrator (Model VC-360, TAITEC, Saitama, Japan) under reduced pressure. The dried materials were dissolved in 100  $\mu$ l of trimethylsilylation reagent consisting of dry pyridine (P57506, Sigma), bistrimethylsilylacetamide (15241-25L, Fluka), and trimethylchlorosilane (C72854-5ML, Aldrich; 3 : 5 : 2, v/v) and were allowed to react at room temperature overnight. The samples were then heated at 90°C for 3 h to complete the derivatization. After centrifugation at 1,500  $\times$  g for 3 min, 2  $\mu$ l of the supernatant were analyzed with a GLC (Model GC-14A, Shimadzu, Kyoto, Japan) equipped with a hydrogen flame-ionization detector (FID). Trimethylsilylated derivatives were separated on a column (1.5% silicone OV-17 on Shimalite W, 3.2 mm  $\times$  2.1 m, Wako Pure Chemical Industries). Temperatures of the injection port and FID were 270°C and 280°C, respectively. Nitrogen was used as a carrier gas at a flow rate of 70 ml min<sup>-1</sup>.

**Measurement of photosynthetic O<sub>2</sub> evolving activity.** The photosynthetic O<sub>2</sub> evolution activity of *N. punctiforme* cells was measured using a Clark-type oxygen electrode (Rank Brothers, Ltd., Cambridge, UK) using an assay medium of 25 mM HEPES-NaOH (pH 7.0) containing 10 mM NaHCO<sub>3</sub> as a final electron acceptor under saturated actinic light of 1,600  $\mu$ E m<sup>-2</sup> s<sup>-1</sup> at 30°C. After measurement of the initial level of O<sub>2</sub> evolution, the cells were air-dried. The air-dried cells were rehydrated in 25 mM HEPES-NaOH (pH 7.0) to mea-

sure the remaining activity of O<sub>2</sub> evolution.

**Determination of chlorophyll *a* content.** Chlorophyll *a* (Chl *a*) was extracted from the cells with 100% methanol. The concentration of Chl *a* was calculated from A<sub>665</sub> with an extinction coefficient of 71.942 L g<sup>-1</sup> cm<sup>-1</sup> (Tandeu de Marsac and Houmard, 1988).

**Vital staining of cells with fluorescein diacetate (FDA).** FDA was used to stain live cells. The cells were observed by fluorescence microscopy as described (Tamaru et al., 2005). The survival rate was calculated from the number of cells emitting green fluorescence normalized to the total cell number.

**Detection of mRNA by RT-PCR.** The DNA fragments covering the 7-kb region of the *treZYH* genes of *N. punctiforme* strain IAM M-15 encoding Mth, Mts, and trehalase, respectively, were amplified by PCR using specific primers designed from the nucleotide sequence of the *N. punctiforme* strain ATCC 29133 genome (Meeks et al., 2001). The nucleotide sequence of the *treZYH* genes of *N. punctiforme* strain IAM M-15 was determined and was submitted to DDBJ/EMBL/GenBank under accession number AB439289.

RT-PCR analysis was performed to detect the transcripts of the *treZ*, *treY*, and *treH* genes during desiccation. As a control, the transcripts for the *groEL* gene encoding chaperonin 60 were used, since GroEL is an abundant protein in natural colonies of *N. commune*; mRNA for the *groEL* gene is detected at similar levels by RT-PCR from both hydrated and desiccated cells (Yoshiyuki Tamaru and Toshio Sakamoto, unpublished data). The first-strand cDNA was synthesized from total RNA isolated from the *N. punctiforme* cells with AMV reverse transcriptase (TaKaRa RNA PCR Kit, TaKaRa, Shiga, Japan). Using the cDNA template and the specific PCR primers for the *treZ* gene (forward, 5'-ATATTATCAAGATTTTGGGC; reverse, 5'-TTGCTTCGATGAAATTGCGG), the *treY* gene (forward, 5'-CTTACACAAAATTTATCACC; reverse, 5'-TGACCGG-GATTTCCCGGAC), the *treH* gene (forward, 5'-CGCAACCCCTGTCCTCAGC; reverse, 5'-GCAAA-TTCGTAGCAACGGCG), and the *groEL* gene (forward, 5'-CTTGAACGAGGCATTGACA; reverse, 5'-TTGT-CAACACCATACCAGCG), the DNA fragments were amplified by PCR, fractionated by agarose electrophoresis, and stained with ethidium bromide.

**Preparation of cell-free extracts and enzyme assays.** *Nostoc punctiforme* cells were harvested by centrifugation, and 9 g of cell pellet were frozen using liquid N<sub>2</sub> and ground with a mortar and pestle. The cell

powder was suspended in 100 ml of 100 mM potassium phosphate buffer (pH 7.0) and disrupted by sonication. After centrifugation at 22,000 × *g* at 4°C for 10 min, the supernatant was recovered and ammonium sulfate was added to 20% saturation. The supernatant was then centrifuged at 22,000 × *g* at 4°C for 10 min, and ammonium sulfate was added to the secondary supernatant to increase its saturation to 50%. After centrifugation at 22,000 × *g* at 4°C for 10 min, the pellet was dissolved in 20 mM potassium phosphate buffer (pH 7.0) and dialyzed against 20 mM potassium phosphate buffer (pH 7.0) overnight. The dialysate was then subjected to centrifugation at 22,000 × *g* at 4°C for 10 min, and the supernatant was used as the cell-free extract for the enzyme assay.

The activity of trehalose synthesis, consisting of Mts and Mth, was measured by the production of trehalose from soluble starch. The reaction mixture contained 25 mM HEPES-NaOH (pH 7.5), 0.07% soluble starch, and 120 μl of the cell-free extract in a total volume of 1.2 ml. The reaction mixture was incubated at 37°C for 3 h, and the reaction was stopped by boiling for 10 min. After centrifugation at 21,500 × *g* for 10 min, the supernatant was lyophilized, and the trehalose level was determined by GLC as described above. One unit of activity was defined as the amount of enzyme that catalyzed the synthesis of 1 μmol of trehalose per min.

The activity of trehalase was measured by the production of glucose from trehalose. The reaction mixture contained 25 mM HEPES-NaOH (pH 7.5), 50 mM trehalose, and 30 μl of the cell-free extract in a total volume of 300 μl. The reaction mixture was incubated at 37°C for 0.5 to 3 h, and the reaction was stopped by boiling for 10 min. The level of glucose was determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of activity was defined as the amount of enzyme that catalyzed the production of 1 μmol of glucose per min.

**Expression and purification of recombinant *NpTreH* in *Escherichia coli* cells.** The *treH* gene of *N. punctiforme* strain IAM M-15 was amplified by PCR with the primers 5'-CATATGATCACTCCCCAGC-3' (forward) and 5'-GGATCCTTAACTTTTTTCC-3' (reverse). The amplified 1.5-kb DNA fragment was ligated into the *Sma*I restriction site of pUC19, and the insert DNA was transferred into the *Nde*I/*Bam*HI sites of pET-28a(+) (Novagen, San Diego, California). The resulting plasmid was designated pET*treH*. The nucleotide sequence of the *Nde*I-*Bam*HI fragment encoding *NpTreH*

was submitted to DDBJ/EMBL/GenBank under accession number AB427107. The *E. coli* BL21-KJE8 (DE3) harboring the pG-KJE8 plasmid (Chaperone Plasmid Set, TaKaRa) was transformed with pET*treH* to express *NpTreH* containing a His-tag at the N-terminus. Bacterial cells transformed with pET*treH* were inoculated into 200 ml Luria-Bertani (LB) medium containing 10  $\mu\text{g}$  kanamycin  $\text{ml}^{-1}$  and 35  $\mu\text{g}$  chloramphenicol  $\text{ml}^{-1}$  and grown at 37°C under vigorous aeration to an  $\text{OD}_{600}$  of ca. 0.2. After addition of 2 mg arabinose  $\text{ml}^{-1}$  and 5 ng tetracycline  $\text{ml}^{-1}$ , growth temperature was decreased to 25°C. When the  $\text{OD}_{600}$  reached 0.6, IPTG was added to a final concentration of 0.1 mM. After additional incubation at 25°C for 6 h, the cells were harvested by centrifugation at 22,000  $\times g$  for 10 min, suspended in 4 ml of 100 mM potassium phosphate buffer (pH 7.0), and disrupted by sonication. After ultracentrifugation at 100,000  $\times g$  at 4°C for 10 min, one-tenth volume of 50 mM ATP and 100 mM  $\text{MgSO}_4$  was added to the clear lysate and incubated at 37°C for 30 min to release the recombinant *NpTreH* from the chaperone proteins. The lysate was diluted with ten volumes of binding buffer consisting of 20 mM potassium phosphate buffer (pH 7.0), 0.5 M NaCl, and 20 mM imidazole and applied to a HisTrap HP column (GE Healthcare, Buckinghamshire, England) equilibrated with binding buffer. After washing the column with ten column volumes of binding buffer, the recombinant *NpTreH* was eluted with an elution buffer consisting of 20 mM potassium phosphate buffer (pH 7.0), 0.5 M NaCl, and 0.5 M imidazole. The active fractions were concentrated, and the buffer was exchanged to 100 mM potassium phosphate buffer (pH 7.0) using a Centricon YM-30 unit (Millipore Corporation, Bedford, Massachusetts).

**Protein determination and SDS-PAGE.** Protein concentrations were determined using the Coomassie Brilliant Blue (CBB) G-250 dye-binding method described by Bradford (1976) with BSA as a standard. SDS-PAGE was performed according to the method of Laemmli (1970) using vertical electrophoresis glass plates. Proteins were stained using CBB R-250. Molecular masses were estimated using a molecular size marker set (Precision Plus Protein unstained standards, Bio-Rad Laboratories, Hercules, California).

## Results

### *Changes in trehalose and sucrose levels in response to desiccation*

Trehalose and sucrose levels in desiccated *N. punctiforme* cells were examined (Fig. 1). Trehalose was not detectable ( $<0.3 \mu\text{mol g}^{-1}$  DW) in the untreated cells in which the water content was  $>20$ , suggesting that trehalose can be consumed in fully hydrated and metabolically active cells. During desiccation, trehalose accumulation took place when the water content became  $<5$ , where the relative water content (RWC) as defined by  $(\text{FW}-\text{DW}) / (\text{initial FW}-\text{DW})$  was  $<14\%$ . The air-dried cells of *N. punctiforme* contained  $40.3 \pm 18.1 \mu\text{mol g}^{-1}$  DW ( $N=10$ ) trehalose. Sucrose was detected in the untreated cells and remained detectable throughout the desiccation treatment. The sucrose level of the air-dried cells of *N. punctiforme* was  $10.9 \pm 5.4 \mu\text{mol g}^{-1}$  DW ( $N=10$ ), which was approximately 25% that of trehalose. These results suggest that trehalose accumulation is tightly controlled in response to water loss during desiccation and that this organism accumulates trehalose as a protectant during desicca-

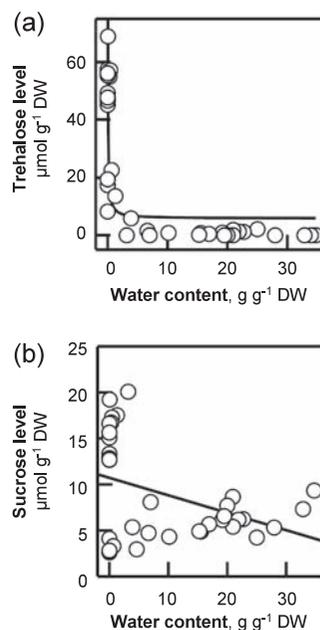


Fig. 1. Changes in trehalose and sucrose levels during desiccation.

The trehalose (a) and sucrose (b) levels in desiccating *N. punctiforme* cells were determined using GLC and plotted as a function of the water content in the cells. The regression equations of the trehalose level and the sucrose level were  $y = 3.915699x^{-1} + 5.377799$  ( $r^2 = 0.5821262$ ) and  $y = -0.1896006x + 10.72077$  ( $r^2 = 0.1508628$ ), respectively.

tion.

To examine the desiccation tolerance in *N. punctiforme* cells, the restored  $O_2$  evolution activity after desiccation treatment was measured. Photosynthetic  $O_2$  evolution in the cells at the mid- to late-exponential growth phase was approximately  $200 \mu\text{mol } O_2 \text{ mg}^{-1} \text{ Chl } a \text{ h}^{-1}$  and 75% of the initial activity was detected when the air-dried cells were rehydrated, indicating that the photosynthetic activity is maintained during desiccation and is recovered upon rehydration. Vital staining with FDA was performed to assess the viability of the cells after desiccation. FDA stained nearly all cells after desiccation; over 4,800 cells were examined in total, and approximately 95% of cells emitted both green fluorescence from fluorescein and red fluorescence from chlorophyll, indicating maintenance of the plasma membrane and preservation of cell viability after desiccation. These results suggest that *N. punctiforme* strain IAM M-15 is strongly tolerant to desiccation.

#### Changes in trehalose and sucrose levels in response to salt-stress treatment

Trehalose accumulation in response to salt-stress treatment was examined (Fig. 2a). Trehalose accumulation occurred 1 h after treatment with 0.2 M NaCl and was also induced by treatment with 1 M NaCl. The level of trehalose reached approximately  $120 \mu\text{mol g}^{-1} \text{ DW}$  24 h after treatment. Sucrose was detected during the salt-stress treatment; its level increased after treatment with 0.2 M NaCl (Fig. 2b). After treatment with 1 M NaCl, the level of sucrose slightly increased but sucrose accumulation was not evident as trehalose accumulation. These data suggest that *N. punctiforme* cells subjected to salt-stress accumulate trehalose and sucrose, and that the mechanism of salt-stress-induced trehalose accumulation may overlap with the mechanism by which these cells respond to desiccation.

#### Detection of mRNA of *treZYH* genes by RT-PCR

The three genes for trehalose metabolism, *treZ* (Mth), *treY* (Mts), and *treH* (trehalase), were found as a gene cluster in a 7.1-kb region of *N. punctiforme* strain IAM M-15. Changes in mRNA levels for these trehalose metabolism genes during desiccation were examined by RT-PCR (Fig. 3). The RT-PCR products for trehalose synthesis genes as well as for trehalase were detected in desiccating cells. In air-dried cells, all transcripts for

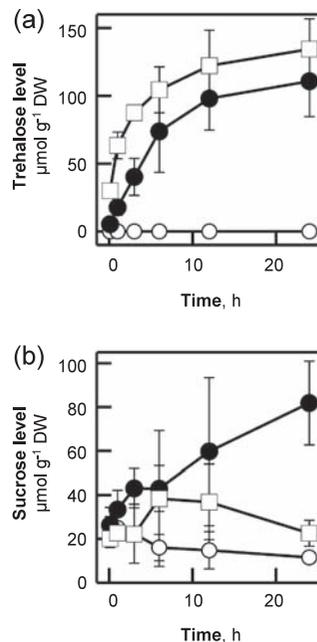


Fig. 2. Trehalose and sucrose accumulation induced by salt-stress treatment.

The *N. punctiforme* cells were treated in 25 mM HEPES-NaOH (pH 7.5) containing 0 M ( $\circ$ ), 0.2 M ( $\bullet$ ), and 1 M ( $\square$ ) NaCl, respectively. Trehalose (a) and sucrose (b) levels were determined using GLC after salt-stress treatment. The experiment was replicated three times, and the averages are shown ( $\pm$  S.D.).

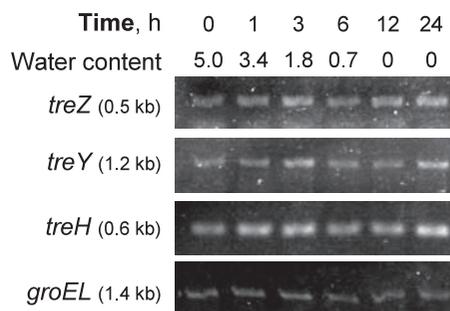


Fig. 3. Detection of mRNA for the *treZ*, *treY*, and *treH* genes by RT-PCR.

The *N. punctiforme* cells were air-dried, and RNA was prepared from the desiccated cells. RT-PCR products with the expected sizes were amplified using specific primers for the *treZ* (Mth), *treY* (Mts), *treH* (trehalase), and *groEL* (chaperonin 60) genes. When RT reactions were omitted to clarify the amplification from RNA templates, no DNA fragment was amplified by PCR (data not shown). The data shown are derived from a single experiment; however, this experiment was repeated twice, and identical results were obtained in both cases.

the *treZ*, *treY*, and *treH* genes were detected 24 h after desiccation treatment. These results suggest that tran-

scriptional up- or down-regulation does not apparently take place in the *treZ*, *treY*, or *treH* genes in response to desiccation and that both genes for trehalose synthesis and trehalose hydrolysis are expressed simultaneously in the desiccated cells. The transcriptional regulation of the gene cluster for trehalose metabolism could not fully explain trehalose accumulation induced by desiccation. Thus, the biochemical properties of Mts + Mth and trehalase were examined further.

#### Biochemical properties of Mts + Mth and trehalase

The enzymatic properties of Mts + Mth and trehalase were characterized in vitro using cell-free extract from *N. punctiforme*. The activity of trehalose synthesis from soluble starch, consisting of Mts and Mth, was detected in the cell-free extract using our assay system. The optimal pH for the activity of trehalose synthesis was 7.0, and it was active in the presence of Tris (Fig. 4a). The activity of trehalose hydrolysis to glucose was also detected in the cell-free extract. The optimal pH for trehalase was 7.5 (Fig. 4b), indicating that it belongs to a neutral trehalase (Thevelein, 1984). The  $K_m$  value of trehalase for trehalose was 18 mM. During the measurement of trehalase pH dependency, the trehalase activity of *N. punctiforme* was strongly inhibited by the presence of Tris. This effect of Tris on trehalase was characterized further (Table 1). Although trehalase activity was detected at pH 7.5 with HEPES buffer or TES buffer, the addition of 5 mM Tris-HCl buffer strongly inhibited trehalase activity. This result indicates that Tris is a strong inhibitor of trehalase, although the manner of inhibition of trehalase activity by Tris is un-

known.

The effects of NaCl on the activities of Mts + Mth and trehalase are shown in Fig. 5. Trehalose produc-

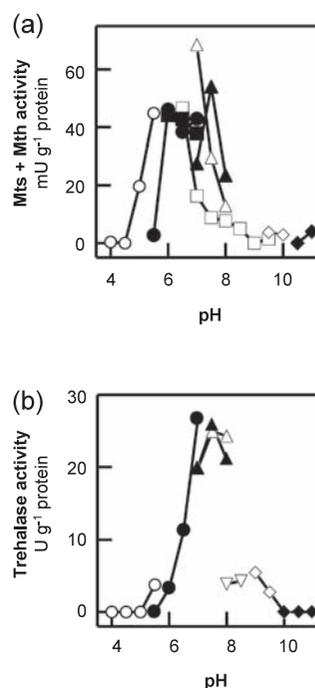


Fig. 4. Effects of pH on the activities of trehalose synthesis and trehalase.

Trehalose synthesis (Mts + Mth) (a) and trehalase activities (b) in cell-free extracts from *N. punctiforme* were measured with several buffered solutions: acetate buffer (pH 4.0 to 5.5, ○), MES-NaOH (pH 5.5 to 7.0, ●), Tris-HCl (pH 6.5 to 9.5, □), Bis-Tris-HCl (pH 6.0 to 7.0, ■), HEPES-NaOH (pH 7.0 to 8.0, △), TES-NaOH (pH 7.0 to 8.0, ▲), Tricine-NaOH (pH 8.0 to 8.5, ▽), CHES-NaOH (pH 9.0 to 10.0, ◇), and CAPS-NaOH (pH 10.0 to 11.0, ◆).

Table 1. Inhibitory effect of Tris on trehalase activity.

Buffering solution	Mts + Mth activity <sup>a</sup> (mU g <sup>-1</sup> protein)	Trehalase activity <sup>b</sup> (U g <sup>-1</sup> protein)	<i>NpTreH</i> activity <sup>c</sup> (kU g <sup>-1</sup> protein)
25 mM HEPES-NaOH (pH 7.5)	30	25	477
25 mM TES-NaOH (pH 7.5)	54	26	446
25 mM Tris-HCl (pH 7.5)	9	< 0.3	< 0.5
25 mM Tris-H <sub>2</sub> SO <sub>4</sub> (pH 7.5)	NT <sup>d</sup>	< 0.3	< 0.5
25 mM HEPES-NaOH + 5 mM Tris-HCl (pH 7.5)	NT	< 0.3	9

Experiments were repeated twice. The mean values are shown.

<sup>a</sup>One unit of activity was defined as the amount of enzyme that catalyzes the synthesis of 1 μmol of trehalose per min from 0.07% soluble starch as a substrate. The detection limit was 0.1 mU g<sup>-1</sup> protein.

<sup>b</sup>One unit of activity was defined as the amount of enzyme that catalyzes the hydrolysis of trehalose to release 1 μmol of glucose per min. The detection limit was 0.3 U g<sup>-1</sup> protein.

<sup>c</sup>One unit of activity was defined as the amount of enzyme that catalyzes the hydrolysis of trehalose to release 1 μmol of glucose per min. The detection limit was 500 U g<sup>-1</sup> protein.

<sup>d</sup>NT, not tested.

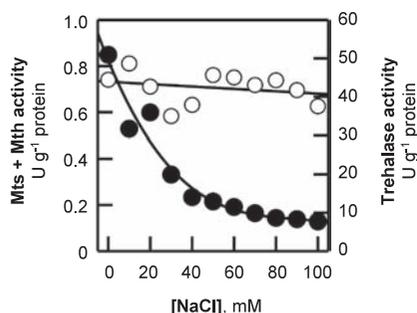


Fig. 5. Effects of NaCl on the activities of trehalose synthesis and trehalase.

The Mts + Mth activities (○) and the trehalase activities (●) in cell-free extracts from *N. punctiforme* were measured in the presence of NaCl and plotted as a function of NaCl concentration in the reaction mixture. The regression equations of the Mts + Mth activity and the trehalase activity were  $y = -0.0005153x + 0.7317336$  ( $r^2 = 0.064$ ,  $p = 0.453$ ) and  $y = -0.000056x^3 + 0.006266x^2 - 0.2768524x + 26.812972$  ( $r^2 = 0.949$ ,  $p < 0.0001$ ), respectively.

tion from soluble starch was insensitive to NaCl and remained active in the presence of 100 mM NaCl. Trehalase activity decreased as NaCl concentration increased, and 75% of the activity was inhibited in the presence of 50 mM NaCl. Trehalase activity was also inhibited by 10 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, and 20 mM MgSO<sub>4</sub>; trehalase activity was decreased to less than 10% of the activity of the control in the presence of these divalent cations (data not shown). These results demonstrate that trehalase is strongly inhibited by salts in the reaction mixture while trehalose production is unaffected by salts.

#### Expression, purification, and characterization of recombinant *NpTreH*

Recombinant *NpTreH* produced in *E. coli* was purified to homogeneity by Ni-affinity chromatography (Fig. 6a), and the biochemical properties of the purified 52-kDa *NpTreH* were characterized. The optimal pH was 7.5 (Fig. 6b), which is identical to trehalase in cell-free extracts from *N. punctiforme* (Fig. 4b). The activity of *NpTreH* was strongly inhibited in the presence of 5 mM of Tris (Table 1), although *NpTreH* activity was detected at pH 7.5 with HEPES or TES buffer. The effect of NaCl on activity of *NpTreH* is shown in Fig. 6(c). The activity of *NpTreH* decreased as NaCl concentration increased, and addition of 10 mM NaCl inhibited the activity of *NpTreH* by 75%. These enzymatic properties of *NpTreH* were essentially identical to those of trehalase in cell-free extracts from *N. punctiforme*.

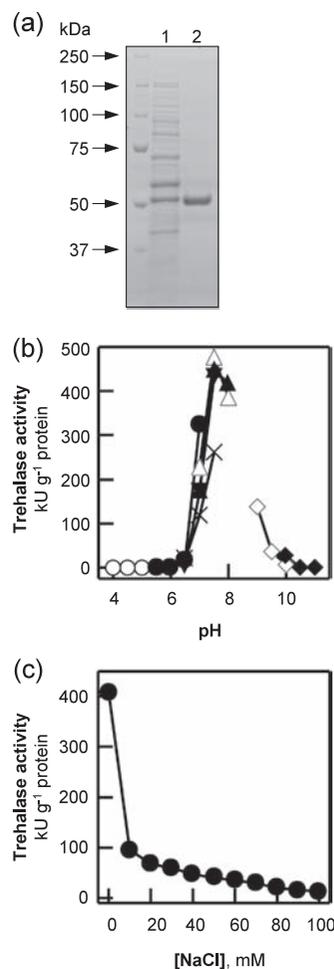


Fig. 6. Purification and characterization of recombinant *NpTreH*.

(a) Expression and purification of recombinant *NpTreH*. The crude extract of *E. coli* that expressed *NpTreH* contained 16 mU of trehalase activity (lane 1). His-tagged *NpTreH* that was purified using nickel chelating resin and that contained 1.2 U of trehalase activity (lane 2) was fractionated by SDS-PAGE and stained with CBB. (b) Effect of pH on the activity of recombinant *NpTreH*. The activity of the purified *NpTreH* was measured in the presence of several buffered solutions: acetate buffer (pH 4.0 to 5.5, ○), MES-NaOH (pH 5.5 to 7.0, ●), MOPS-NaOH (pH 6.5 to 7.5, ▼), PIPES-NaOH (pH 6.5 to 7.5, ×), HEPES-NaOH (pH 7.0 to 8.0, △), TES-NaOH (pH 7.0 to 8.0, ▲), CHES-NaOH (pH 9.0 to 10.0, ◇), and CAPS-NaOH (pH 10.0 to 11.0, ◆). (c) Effect of NaCl on the activity of recombinant *NpTreH*. The activities of the purified *NpTreH* were measured in the presence of NaCl and plotted as a function of NaCl concentration in the reaction mixture.

Since the *NpTreH* was purified and no other proteins were detected (Fig. 6a), other cellular proteins of *N. punctiforme* were unlikely to be involved in the regulation of trehalase activity. These results suggest that

trehalase encoded by the *treH* gene is a key enzyme that controls trehalose accumulation in response to water-stress conditions in *N. punctiforme*.

## Discussion

In cyanobacteria, a close correlation has been shown between major accumulated compatible solutes and the range of salt tolerance (Blumwald et al., 1983; Borowitzka et al., 1980; Mackay et al., 1984; Reed et al., 1984). Cyanobacteria are classified into three groups of salt tolerance depending on the types of osmoprotective compounds. Strains with low salt tolerance (up to 0.7 M) synthesize trehalose and/or sucrose. Strains with moderate salt tolerance (up to 1.8 M) use glucosylglycerol, and strains with the highest salt tolerance (up to 2.5 M) accumulate glycine betaine or glutamate betaine. *Nostoc commune* has low salt tolerance and field-isolated, natural colonies of *N. commune* accumulate trehalose in response to matrix water stress (desiccation) and osmotic water stress (high salt concentrations; Sakamoto et al., 2009). *Nostoc punctiforme* strain IAM M-15 cells also accumulate trehalose in response to both matrix and osmotic water stress (Figs. 1a and 2a). This is consistent with the idea that some acclimatizing mechanisms may be common to both matrix and osmotic water stress (Hayashi and Murata, 1998). It is noteworthy that trehalose is less effective as an osmoprotective compound, but is relevant to the extreme desiccation tolerance observed in terrestrial cyanobacteria.

Although sucrose accumulation as a compatible solute has been reported in several cyanobacteria (Figs. 1b and 2b; Reed and Stewart, 1985), the trehalose accumulation capacity may be relevant to the tolerance of the extremely desiccating environment of terrestrial cyanobacteria. As *N. punctiforme* cells contained approximately 5.7 mg Chl g<sup>-1</sup> DW, the amount of trehalose normalized to chlorophyll levels is 2.4  $\mu\text{g } \mu\text{g}^{-1}$  Chl. Trehalose accumulation in *N. punctiforme* is consistent with that observed in field-isolated, natural colonies of *N. commune* (approximately 1.4  $\mu\text{g } \mu\text{g}^{-1}$  Chl; Sakamoto et al., 2009) and is in good agreement with drought-resistant cyanobacteria, *Phormidium autumnale* and *Chroococciopsis* sp., which accumulate trehalose in response to matrix water stress up to 6.2  $\mu\text{g}$  and 3.2  $\mu\text{g } \mu\text{g}^{-1}$  Chl, respectively (Hershkovitz et al., 1991). In the freshwater strain of the cyanobacterium *Nostoc* sp. PCC 7120, which is

not tolerant of extreme desiccation, sucrose is accumulated under dehydrating conditions to approximately 0.8  $\mu\text{g } \mu\text{g}^{-1}$  Chl but the trehalose level is only 0.03  $\mu\text{g } \mu\text{g}^{-1}$  Chl (Higo et al., 2006). In the mutant of *Nostoc* sp. PCC 7120 with disrupted *treH* gene, the amount of trehalose accumulated during dehydration increases to 0.05  $\mu\text{g } \mu\text{g}^{-1}$  Chl and the dehydration tolerance is enhanced (Higo et al., 2006). In *N. punctiforme*, sucrose was detected in non-stressed cells and during water stress treatments and the change in sucrose content was smaller than that in trehalose (Figs. 1b and 2b). The sucrose level detected in *N. punctiforme* cells (approximately 0.6  $\mu\text{g } \mu\text{g}^{-1}$  Chl) is roughly equivalent to that in *Nostoc* sp. PCC 7120. These data indicate that *N. punctiforme* has a similar capacity of sucrose accumulation and approximately 80 times more capacity to accumulate trehalose compared to the freshwater strain of the cyanobacterium. These findings suggest that the accumulation of trehalose is important to withstand desiccation. Although it has been proposed that trehalose protects proteins and membranes in bacteria during drying (Leslie et al., 1995), the protective mechanism of trehalose in cyanobacteria, in which the photosynthetic apparatus should be protected in the desiccated state, remains to be elucidated.

In the filamentous cyanobacterium *Nostoc* sp. PCC 7120, the genes *treZ*, *treY*, and *treH*, encoding Mth, Mts, and trehalase, respectively, constitute a transcriptional unit (Higo et al., 2006). This cluster of genes is conserved among cyanobacteria, suggesting that the simultaneous transcriptional regulation of *treZ*, *treY*, and *treH* is important for water-stress-induced gene expression. However, the accumulation of trehalose is likely not explicable by the mere transcriptional regulation of these genes, since the transcriptional up-regulation of trehalase appears odd for an acclimatizing response to desiccation. Therefore, we investigated the biochemical properties of the enzymes involved in trehalose metabolism in *N. punctiforme* strain IAM M-15 to elucidate the mechanisms of trehalose accumulation in *Nostoc*. The activities of trehalose production and trehalase were detected in cell-free extracts from *N. punctiforme* (Fig. 5). Trehalase activity was strongly inhibited in the presence of Tris, and Tris did not affect trehalose synthesis activity (Table 1). Based on this observation, we postulated that a potent trehalase-specific inhibitor with a small molecular weight may be involved in the control of trehalase activity dur-

ing desiccation. A natural inhibitor for trehalase was sought using a concentrated boiling-water extract of *Nostoc*, but this inhibitor was not found (data not shown). Since trehalase activity was inhibited by the presence of 10 mM NaCl (Fig. 6c), water-stress conditions in which the cellular concentration of solutes increase should also inhibit the enzyme. Therefore, it stands to reason that the rate of trehalose production exceeds that of trehalose hydrolysis under water-stress conditions. Upon rehydration, trehalase can be reactivated when the intracellular concentration of solutes decreases, and trehalose can be consumed as a respiratory substrate in the metabolically active cells. Although this model explains trehalose accumulation under water-stress conditions in this organism, it remains to be demonstrated whether trehalase is regulated in this manner in vivo.

In bacteria and yeast, trehalose rapidly accumulates in large quantities under stresses such as high salt, osmosis, drought, heat, and cold (De Virgilio et al., 1994; Hottiger et al., 1987, 1989; Kandror et al., 2002; Wolf et al., 2003). Increases in de novo synthesis of trehalose are responsible for the accumulation of trehalose, and synthesis of trehalose mainly depends on the TreY-TreZ pathway or/and the trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) pathway (De Virgilio et al., 1994; Elbein et al., 2003; Kandror et al., 2002; Wolf et al., 2003). An *E. coli* mutant that is unable to produce trehalose shows little protection against low temperatures compared to wild-type cells, and trehalose levels and cell viability is restored by transformation with *otsA* and *otsB* genes encoding TPS and TPP, respectively (Kandror et al., 2002). In yeast, inactivation of either TPS1 or TPS2 results in an inability to accumulate trehalose upon mild heat shock, and this significantly reduced heat-induced thermo-tolerance (De Virgilio et al., 1994). Interestingly, trehalose accumulation and increases in TPP activity are induced by salt stress in the cyanobacterium *Scytonema* sp. isolated from desert soils (Page-Sharp et al., 1999). Nevertheless, the mechanism of the up-regulation of trehalose synthesis via the TPS-TPP pathway has not been characterized in cyanobacteria.

In fungi, trehalases can be divided into two groups. The first group contains trehalases with an acid pH optimum (pH 3.5 to 5.5), and the second group has a neutral pH optimum (pH 6 to 7.5). The neutral trehalase regulates trehalose mobilization by rapidly changing

its activity in response to environmental stimuli and is controlled by cAMP-dependent protein phosphorylation. It is therefore called "regulatory trehalase" (Thevelein, 1984). In *Saccharomyces cerevisiae*, the neutral trehalase can be post-translationally activated by cAMP-dependent phosphorylation (App and Holzer, 1989; Uno et al., 1983), and the fluctuations of the trehalose pool during temperature shifts are inversely correlated with the activity of neutral trehalase (De Virgilio et al., 1991). This suggests that neutral trehalase plays a key role in the regulation of the trehalose pool in yeast. Unlike *Schizosaccharomyces pombe* (Soto et al., 1998), trehalase of *N. punctiforme* has no consensus site for cAMP-dependent phosphorylation. Hence, it is unlikely that cyanobacterial trehalase is controlled by phosphorylation.

In conclusion, control of trehalase plays an important role in trehalose accumulation in terrestrial cyanobacteria under conditions of extreme desiccation. Two questions remain to be answered in future studies: (1) whether control of trehalase activity is a general model for trehalose accumulation; and (2) how *Nostoc* trehalase is controlled in vivo.

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