J. Microbiol. Biotechnol. (2011), **21**(8), 830–837
doi: 10.4014/jmb.1101.10068
First published online 5 June 2011

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Molecular Cloning of Maltooligosyltrehalose Trehalohydrolase Gene from *Nostoc flagelliforme* and Trehalose-Related Response to Stresses

Wu, Shuangxiu[†], Liang He[†], Rongrong Shen, Xiu Zhang, and Quanxi Wang[*]

Department of Biology, College of Life and Environmental Science, Shanghai Normal University, Guilin Road 100, Shanghai, 200234, People's Republic of China

Received: January 13, 2011 / Revised: May 6, 2011 / Accepted: May 25, 2011

A genomic DNA fragment encoding a putative maltooligosyltrehalose trehalohydrolase (NfMTH) for trehalose biosynthesis was cloned by the degenerate primer-PCR from cyanobacterium *Nostoc flagelliforme*. The ORF of *NfMTH* is 1,848 bp in length and encodes 615 amino acid residues, constituting a 70 kDa protein. The deduced amino acid sequence of *NfMTH* contains 4 regions highly conserved for MTHs. By expression of *NfMTH* in *E. coli*, the function of this protein was demonstrated, where the recombinant protein catalyzed the hydrolysis of maltooligosyl trehalose to trehalose. The expressions of MTH and maltooligosyltrehalose synthase in the filaments of *N. flagelliforme* were upregulated significantly under dehydration stress, NaCl stress, and high temperature-drought stress. The accumulations of both trehalose and sucrose in the filaments of *N. flagelliforme* were also improved significantly under the above stresses. Furthermore, trehalose accumulated in smaller quantities than sucrose did when under NaCl stress, but accumulated in higher quantities than sucrose did when under temperature-drought stress, indicating that both trehalose and sucrose were involved in *N. flagelliforme* adapted to stresses and different strategies conducted in response to various stress conditions.

Keywords: *Nostoc flagelliforme*, maltooligosyltrehalose trehalohydrolase, degenerate primer, desiccation stress, NaCl stress, temperature stress

Nostoc flagelliforme is a kind of terrestrial and macroscopic cyanobacterium distributed in the northern and west-northern parts of Asia, Europe, and USA [5, 8]. It contains high contents of amino acids, and anti-aging and antitumor compounds, and has been used as a food delicacy as well as an herbal medicine ingredient by the Chinese for centuries [8]. In nature, *N. flagelliforme* showed marked desiccation tolerance and extreme heat resistance. It usually grows on dry desert steppes, bare lands, or hills in dry or semi-dry areas where it often experiences severe environment changes such as severe drought, high temperature, and poor and alkaline soil, under which other terrestrial *Nostoc* species hardly survive [8]. Its filaments dried for a couple of years could recover metabolic activity within hours after rehydration [5, 9]. Its photosynthetic carbon fixation was active even under extremely dry conditions and strong solar radiation in July when the land surface temperature was often above 44°C [9]. Many studies have been performed on its morphology, ecology, physiology, and cultivation in China [8, 9], but the molecular mechanism of *N. flagelliforme* adapted to such drought conditions is still poorly understood.

In bacteria, fungi, yeast, invertebrates, and some plants, trehalose was reported to serve as a protective agent and/or carbohydrate reserve under such conditions as drought, heat, salinity, and oxidative stresses, owing to its novel structure of α-D-glucopyranosyl-[1,1]-α-D-glucopyranose [6]. In *N. flagelliforme*, trehalose has been detected, and a *MTS* gene encoding maltooligosyltrehalose synthase for catalyzing maltooligosaccharides to produce maltooligosyltrehalose has been cloned and characterized in our previous work [17, 23]. In this pathway for trehalose biosynthesis, trehalose is produced by a sequential reaction catalyzed by maltooligosyltrehalose synthase (MTS, TreY) and maltooligosyltrehalose trehalohydrolase (MTH, TreZ). MTS first converts α-1,4-glycosidic linkage at the reducing end of maltooligosaccharides into α-1,1 linkage, producing a maltooligosyltrehalose. The trehalose portion of the intermediate is then cleaved by MTH to give trehalose plus a shorter maltooligosaccharide [16, 20, 21]. A group of thermophilic archaebacteria and several mesophilic eubacteria as well as several species of genus *Nostoc* were proved to accumulate trehalose by this pathway, which was briefly reviewed in

*Corresponding author
Phone: +86-21-64322141; Fax: +86-21-64322142;
E-mail: wangqx@shnu.edu.cn

[†]Both these authors contributed equally to this work.

our previous report. In this study, we continued cloning of a genomic DNA fragment encoding a putative maltooligosyltrehalose trehalohydrolase (NfMTH) for trehalose biosynthesis from *N. flagelliforme* by degenerate primer-PCR. The function of the recombinant NfMTH was confirmed by expression in *E. coli*. The expression levels of both MTH and MTS as well as contents of trehalose and sucrose in *N. flagelliforme* were detected during dehydration stress, NaCl stress, and temperature-drought stress. The possible protection mechanism of trehalose in *N. flagelliforme* is discussed.

Materials and Methods

Algal Materials and Sample Preparation

The filament of *N. flagilliforme* was collected from the Inner Mongolia Autonomous Region of China in the summer of 2004 and 2008, and stored in a desiccation cabinet at room temperature before being used for experiments. The filaments were washed 3 times with double distilled water and rehydrated for 24 h before 0.2−0.5 g of the filaments was weighted for DNA and protein extraction or for stress treatments.

Total DNA Extraction and Crude Protein Preparation

Rehydrated filament (0.2−0.5 g) for 24 h or stress-treated filaments of *N. flagilliforme* at the pointed time were ground in liquid nitrogen to powders and then subjected for DNA extraction and crude protein preparation as previously described [23]. About 30 µg of total proteins was added into a 500 µl reaction mixture to conduct the MTH activity assay as previously described.

Cloning of *NfMTH* Gene

The degenerate primers for cloning of the coding region of *NfMTH* from the genomic DNA of *N. flagilliforme* were designed according to the method and reference species reported in our previous work [23]. 5'-GTGA(A,G)AATTGG(T,C)GCT(A,C)ACTACTTGGG-3' was used for the up primer, and 5'-CGATAAGTGGCAATAGGAATTCGCAT-3' for the down primer. The PCR amplification was performed in 50 µl reaction mixtures with the Hot-Start Platinum *Taq* polymerase (Invitrogen, San Diego, CA, USA) for a 30-cycle reaction. In each reaction cycle, the denaturation was at 94°C for 30 s, annealing was at 66.4°C for 1 min, and elongation was at 72°C for 3 min. The PCR products were assayed by electrophoresis in agarose gel and purified with agarose gel DNA purification kits (TaKaRa, Shuzo, Japan).

To construct the plasmid for expression of recombinant *NfMTH* in *E. coli* strain BL21 (Genotype: fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5), the purified clone was ligated into plasmid pET22b (Novagen, Darmstadt, Germany) *via BamHI* and *SalI* sites to form the expressing plasmid pET22bZ.

Extraction and Purification of Recombinant NfMTH in *E. coli*

Expression of the recombinant NfMTH was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 22°C for 6 h in 100 ml of broth of *E. coli* strain BL21. In order to get more soluble recombinant protein, lysozyme and 1% Triton X-100 were added to the extraction buffer. Then the recombinant protein was extracted and analyzed with SDS−PAGE and purified by Ni^{2+}-NTA-agarose affinity chromatography with the AKTA Purifier (GE Healthcare Co. Ltd, UK) as previously reported [23]. Protein was quantified spectrophotometrically using the Bradford method with bovine serum albumin (BSA) as the standard [2].

MTH Activity Assay

The recombinant NfMTH and NfMTS in *E. coli* strain BL21 were extracted and purified according to the above method [23]. MTH activity was measured as follows: First, 20 µg of the purified recombinant NfMTS and 20 µl of 100 mM maltohexaose (Supelco, Bellefonte, USA) in 500 µl of 50 mM phosphate-citric acid buffer (0.2 M Na_3PO_4, 0.1 M citric acid, pH 7.0) were incubated at 50°C for 24 h. In this reaction, MTS converted the reducing terminal α-1,4-linked residue of maltohexaose into an α-1,1-linked intermediate. The reaction was terminated by heating at 100°C for 10 min and the supernatant was collected. Second, an aliquot of 50 µl supernatant was subjected to HPLC analysis and another aliquot of 500 µl supernatant was incubated with 30 µg of the purified recombinant NfMTH at 55°C for 12 h followed by heating at 100°C for 10 min to terminate the reaction. In this reaction, the trehalose portion of the intermediate was cleaved by MTH to give a trehalose plus a shorter maltooligosaccharide, which was maltotetraosyl trehalose in this report. The reducing sugar and trehalose were analyzed by high-performance liquid chromatography (HPLC) as described below. One unit of the MTH activity was defined as the amount of enzyme required for the reaction to convert 1 µmol of maltotetraosyl trehalose (or maltohexaose) into trehalose in one minute under the above conditions.

Stress Treatment

For drought stress treatments, the 24 h rehydrated filaments were exposed in the air at room temperature for 0 h (control), 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h. For salt stress treatment, the rehydrated filaments were exposed to NaCl solution of various concentrations of 0 mM (control), 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, and 175 mM, respectively, at room temperature for 4 h. For temperature and drought stress treatments, the rehydrated filaments were exposed to air at various temperatures of 4°C, 10°C, 25°C (control), 37°C, and 42°C, respectively, for 4 h. Then the filaments were collected for extraction of crude protein.

Sugar Extraction and Determination in the Filaments

Approximately 0.2 g of rehydrated filament or stress-treated filament of *N. flagelliforme* was ground with 1.5 ml of 100% ethanol and held at 4°C for 4 h for sugar extraction. The supernatant was collected, volatilized, and resuspended in 100 µl of 100% ethanol for HPLC detection (Waters, 600 Controller, USA) equipped with a ZORBAX-NH_2 column (4.6×250 mm, pore size 5 µm; Agilent, USA) and an evaporative light scattering detector (Polymer Laboratories, PL-ELS 2100) as previously reported [23]. The eluent was acetonitrile−water [75:25 (v/v)] at 1.0 ml/min constant flow velocity for 20 min, and the column temperature was 30°C. The trehalose peak (in standard or in sugar extracts) was also confirmed by digestion with trehalase (Sigma, Germany), which converts trehalose to glucose [18].

Western Blotting

About 5 µg of the crude soluble protein samples was separated by using SDS polyacrylamide gel electrophoresis (SDS−PAGE) with

8% in separating gels and 5% in collecting gels, according to the standard protocol. Proteins were transferred to the polyvinylidene fluoride (PVDF; Millipore, USA) membrane and the MTS and MTH proteins were detected by polyclonal anti-MTS and anti-MTH antibodies (made by Shanghai Immune Biotech Co. Ltd., China). Chemilluminescence was detected by an Amersham Enhanced Chemiluminescent (ECL) Plus Western Blotting Detection System (GE Healthcare Co. Ltd., UK) on X-ray film. The optical density from Western blot was conducted by a Gel Image System (GE Healthcare Co. Ltd., UK), with the blank region in the film as the negative control.

RESULTS

Cloning of *NfMTH* Gene from *N. flagilliforme*

Trehalose was detected, and a *MTS* gene encoding maltooligosyltrehalose synthase for catalyzing maltooligosaccharides to produce maltooligosyltrehalose had been cloned and characterized in *N. flagelliforme* in our previous work [16, 22]. *N. punctiforme* and *Anabaena* sp., the close-related species of *N. flagelliforme*, were reported to have the MTS-MTH trehalose biosynthesis pathway. Thus, we designed degenerate primers according to the amino acid sequences of *MTH* of the above species and considering the codon bias in cyanobacteria. A genomic DNA fragment coding the ORF region of *MTH* (*NfMTH*) was cloned from *N. flagelliforme* by the PCR method using the degenerate primers (Fig. 1). The sequencing results showed that the ORF region of *NfMTH* is 1,848 bp in length and encodes 615 amino acid residues with about a 70 kDa protein. The nucleotide sequence of the *NfMTH* gene has been deposited to the GenBank database under the accession number HM802214. The deduced amino acid sequence of NfMTH was compared with the homologous enzymes from *N. punctiforme* (NpMTH), *N. punctiforme* 73102 (NpMTH73102), *Anabaena* PCC 7120 (A7120MTH), *A. variabilis* ATCC 29413 (A29413MTH), *Cyanothece* sp. PCC7424 (Cy7424MTH), and *Syntrophobacter fumaroxidans* (Cy8802MTH). NfMTH showed as high as 92% identity with NpMTH, 90% identity with NpMTH73102,

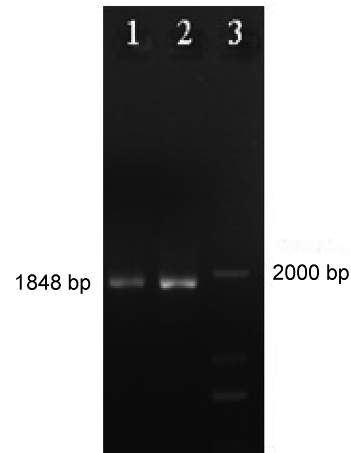

Fig. 1. Electrophoresis assay for identification of *NfMTH* cloned from genomic DNA of *N. flagelliforme* by PCR.
Lanes 1 and 2: PCR product, about 1,848 bp, from genomic DNA of *N. flagelliforme* DNA. Lane 3: molecular marker.

79% identity with A7120MTH and A29413MTH, 45% identity with Cy7424MTH, and 40% identity with Cy8802MTH (Table 1). The multiple sequence alignment revealed four regions highly conserved in the active sites of MTHs (Table 1). Four proposed catalytic residues, His (H), Asp (D), Glu (E), and Asp(D) in conserved regions 1, 2, 3, and 4, respectively (shown in boldface in Table 2), existing in the active sites of α-amylase family enzymes (underlined in Table 2) [7, 12, 14], are also highly conserved in the amino acid sequences of NfMTH.

Purification and Activity of Recombinant NfMTH Enzyme

The expression of recombinant NfMTH was induced by 0.5 mM IPTG from the vector pET22bZ in *E. coli* strain BL21 (DE3) pLysS. The recombinant protein was demonstrated to be mainly insoluble and the molecular mass, confirmed by SDS−PAGE assay, was approximately 75 kDa, including the hexahistidine domain from the pET22bZ vector (Fig. 2). This value is in agreement with the value predicted from its deduced amino acid sequence.

Table 1. Comparison of the deduced amino acid sequences of maltooligosyltrehalose trehalohydrolase (MTHs) from *Nostoc flagelliforme* with those of other MTHs from cyanobacterial genus.

Souce	Accession number	No. of residues	Identity (%)
N. flagelliforme	HM802214.1	1,848	--
N. punctiforme	BAG85336.1	1,848	92
N. punctiforme 73102	YP_001868949.1	1,848	90
Anabena. PCC 7120	NP_484212.1	1,860	79
A. variabilis ATCC 29413	YP_321946.1	1,818	79
Cyanothece sp. PCC7424	YP_002379851.1	1,824	45
Cyanothece sp. PCC8802	YP_003889479.1	1,821	40

The deduced amino acid sequences were obtained from the NCBI GenBank protein sequence database. The identity values were analyzed by the NCBI Blast program on the Web site http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Table 2. Comparison of conserved amino acid sequences in the active sites of α-amylase family enzymes.

		Region 1	Region 2	Region 3	Region 4
A	Generalized sequence	XDXXXNH	GXRXDXXZZ	XXXOEZZZ	XXBBHD
B	MTHs	*******	*******:.	*******	. .****
	NfMTH	201LDVVYN**H**	263GLRL**D**AIQA	300HLIA**E**SDL	453CIQNH**D**
	NpMTH	201LDVVYN**H**	263GLRL**D**AIQA	300HLIA**E**SDL	453CIQNH**D**
	NpMTH73102	201LDVVYN**H**	263GLRL**D**AIQA	300HLIA**E**SDL	453CIQNH**D**
	A7120MTH	215LDVVYN**H**	277GLRL**D**AIQA	314HLIA**E**SDL	467CIQNH**D**
	A29413MTH	201LDVVYN**H**	263GLRL**D**AIQA	300LLIA**E**SDL	453CIQNH**D**
	Cy7424MTH	183LDVVYN**H**	245GLRL**D**AIHA	282YLIA**E**SDL	435CIQNH**D**
	Cy8802MTH	182LDVVYN**H**	244GLRL**D**AIHG	281YLIA**E**SDL	434YAQNH**D**

Row A: the generalized sequences of four conserved regions [23]. X: hydrophobic residue; B: hydrophilic residue; O: Gly or Ala residue; Z: residue important for specificity. Row B: Maltooligosyltrehalose trehalohydrolase enzymes (MTHs). The identical residues among these MTHs are marked by asterisks. The four proposed catalytic residues of His, Asp, Glu, and Asp in regions 1, 2, 3, 4, respectively, are shown in boldface. The residues existing in the active site of NfMTH are underlined. NfMTH: MTH of *N. flagelliforme*; NpMTH: MTH of *N. punctiforme*; NpMTH 73102: MTH of *N. punctiforme* 73102; A7120MTH: MTH of *Anabaena* PCC7120; A29413MTH: MTH of *A. variabilis* ATCC 29413; Cy7424MTH: MTH of *Cyanothece* sp. PCC7424; Cy8802MTH: MTH of *Cyanothece* sp. PCC8802.

The soluble protein product of pET22bZ was purified by Ni^{2+}-NTA-agarose affinity chromatography (Fig. 2, lane 7). The purified recombinant NfMTH was quantified and incubated with the reaction products of the purified recombinant NfMTS and maltohexaose (G6) at 55°C for 12 h. In the reaction of the purified recombinant NfMTS and G6 (reaction I), MTS exerted a glucosyltransferase activity at the reducing end of G6 to convert the first α-1,4-glycosidic linkage into α,α-1,1 linkage, producing an intermediate maltotetraosyl trehalose (G6T). In the reaction of the purified recombinant NfMTH and the products of

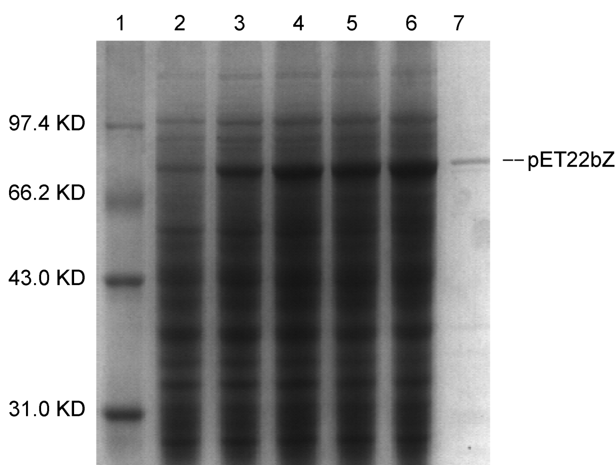

Fig. 2. Expression and purification of the recombinant NfMTH proteins in *E. coli* strain BL21.

Lane 1, molecular weight standards; Lane 2, total protein of *E. coli* without induction; Lanes 3, 4, 5, and 6, insoluble protein of *E. coli* induced by 0.5 mM IPTG at 22°C for 1 h, 2 h, 4 h, and 6 h respectively; Lane 7, purified protein by Ni^{2+}-NTA-agarose affinity chromatography used for enzyme assay. The separation gel of SDS−PAGE was with 8% polyacrylamide and stained with Coomassie Brilliant Blue.

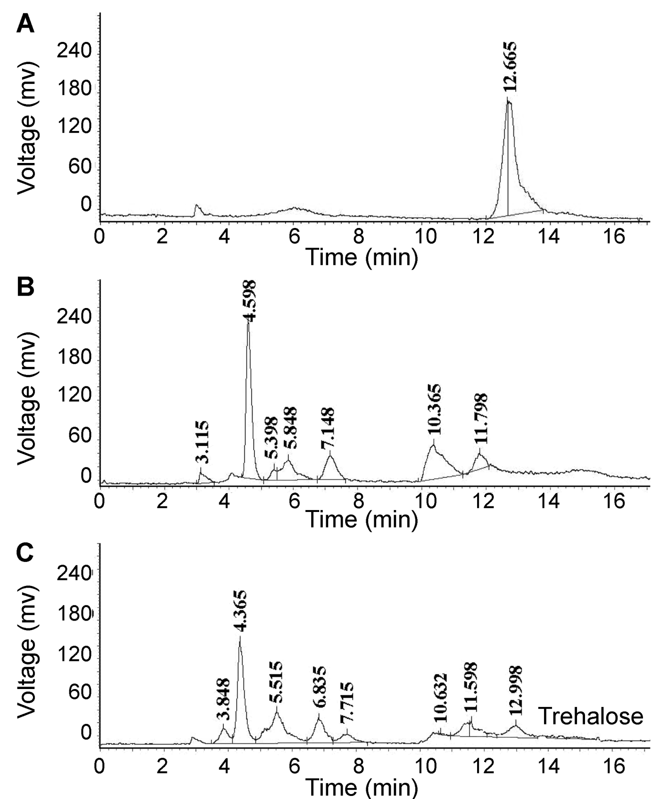

Fig. 3. Trehalose-producing activity of the recombinant NfMTH examinted by HPLC.

A. The profile of trehalose standard. **B**. The profile of the reaction product of the recombinant NfMTS with maltohexaose as the substrate. In this reaction, the recombinant NfMTS converted the first α-1,4-glycosidic linkage of maltohexaose into α-1,1 linkage, producing the intermediate maltotetraosyl trehalose (G6T). **C**. The profile of the reaction product of the recombinant NfMTH incubated with the product of the recombinant NfMTS and maltohexaose. In this reaction, the recombinant NfMTH hydrolyzed the α-1,4-glycosidic bond just near to the α,α-1,1 linkage of G6T to produce a trehalose. For each reaction, the enzyme mixture was heat-denatured for 10 min to terminate the reaction.

reaction I (reaction II), MTH conducted a hydrolysis function to hydrolyze the α-1,4-glycosidic bond just near to the α,α-1,1 linkage of G6T to produce a trehalose and a shorter maltooligosaccharide, a maltotetraose (G4). Both reactions were stopped by boiling for 10 min, respectively, and the corresponding reaction mixtures were examined by HPLC (Fig. 3). The control reaction was set as the purified recombinant NfMTS incubated with G6 followed by adding the glucoamylase to hydrolyze the second α-1,4-glycosidic linkage of the intermediate (G6T) to release glucose and one molecule of trehalose, as reported previously [23]. The results showed that the recombinant enzymes NfMTS and NfMTH together could catalyze the conversion from maltooligosaccharide to produce trehalose, confirming that this recombinant protein had the function of MTHase (Fig. 3C). The activity of recombinant NfMTH was about 0.08 U/mg protein.

Expressions of NfMTS and NfMTH and Accumulation of Trehalose and Sucrose During NaCl and Temperature Stresses

In the metabolism pathway of trehalose in *N. flagelliforme*, both NfMTS and NfMTH conduct the synthesis function. In our previous work, the expression of NfMTS was upregulated and the accumulation of trehalose and sucrose was improved during the dehydration process [23]. In this work, the Western blot analysis revealed that the expression of NfMTH was also upregulated significantly within 4–12 h during the dehydration process and then kept constant up to 24 h (Fig. 4). Together with our previous data on wet weight changes, trehalose and sucrose accumulation, and expression of NfMTS during dehydration stress [22], the result confirmed that for the thin filaments of *N. flagelliforme*, rapid physiological adaptation to the stresses happened in the initial several hours.

In addition, the expression levels of NfMTS and NfMTH and the contents of trehalose and sucrose of the filaments of *N. flagelliforme* during NaCl stress (Fig. 5) and temperature-drought stress (Fig. 6) were also measured. For the rehydrated filaments of *N. flagelliforme* exposed to 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, and 150 mM NaCl stresses for 4 h, the expression levels of NfMTS and NfMTH were significantly upregulated (Fig. 5A and 5B)

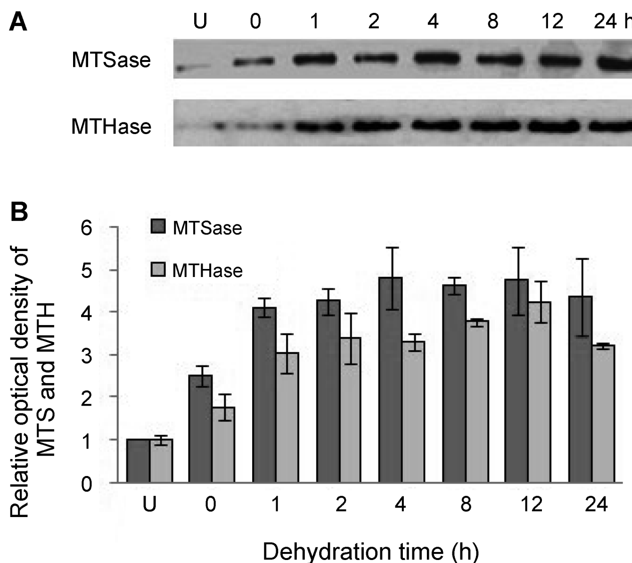

Fig. 4. Expressions of NfMTS and NfMTH in filaments of *N. flagelliforme* during dehydration stress.

The filaments was dried in air at room temperature for up to 24 h. NfMTH and NfMTS expression levels were detected by the specific anti-NfMTS and anti-NfMTH, respectively, at the indicated time during the dehydration process (**A**) and relative expression levels were calculated (**B**). Crude protein (5 μg) was used for the Western blotting analysis and experiments were done at least in triplicates.

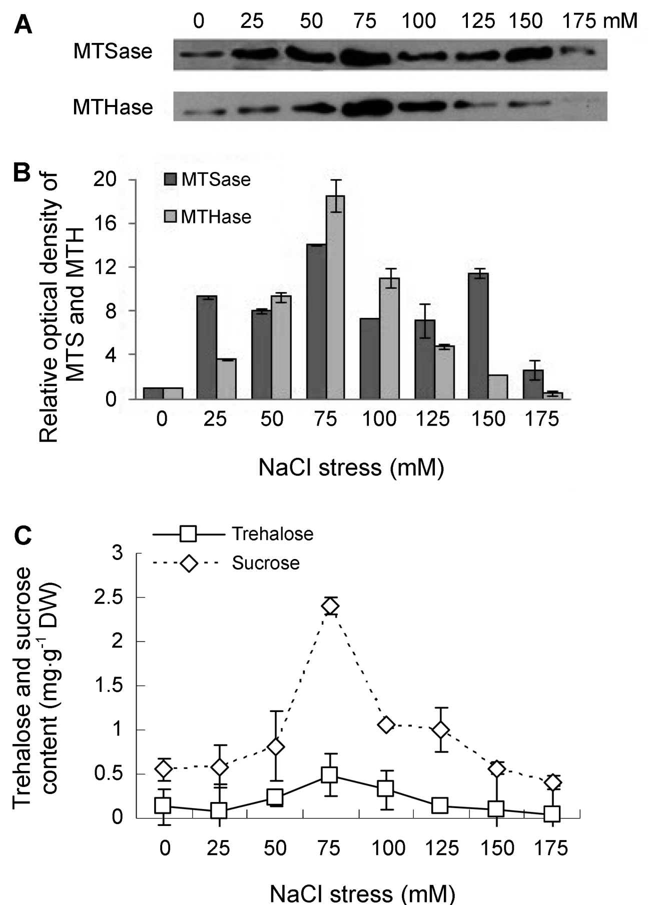

Fig. 5. Expressions of NfMTS and NfMTH and accumulations of trehalose and sucrose in filaments of *N. flagelliforme* during 25–175 mM NaCl stresses for 4 h.

NfMTH and NfMTS expression levels were detected by the specific anti-NfMTS and anti-NfMTH, respectively, at the indicated concentration of NaCl treatment (**A**) and relative expression levels were calculated (**B**). Crude protein (5 μg) was used for the Western blotting analysis. Trehalose and sucrose contents (**C**) were detected by HPLC. All the experiments were done at least in triplicates.

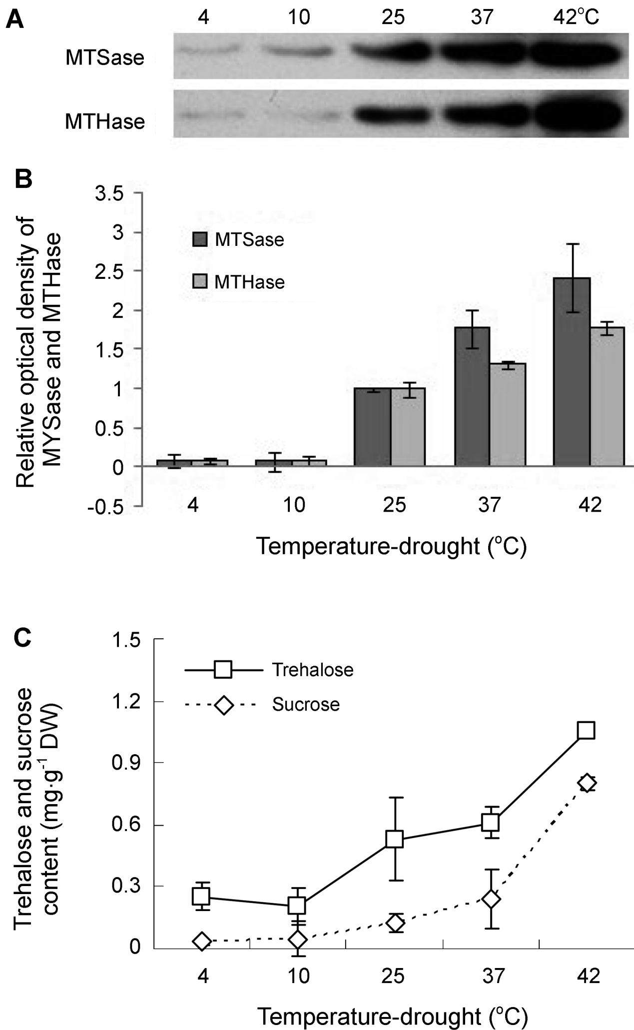

Fig. 6. Expressions of NfMTS and NfMTH and accumulations of trehalose and sucrose in filaments of *N. flagelliforme* during temperature-drought treatments for 4 h.

NfMTH and NfMTS expression levels were detected by the specific anti-NfMTS and anti-NfMTH, respectively, at the indicated temperature treatments (**A**) and relative expression levels were calculated (**B**). Crude protein (5 μg) was used for the Western blotting analysis. Trehalose and sucrose contents (**C**) were detected by HPLC. All the experiments were done at least in triplicates.

and contents of trehalose and sucrose obviously increased (Fig. 5C). The highest upregulation of NfMTS and NfMTH appeared, about 14-fold and 18-fold increased respectively, when the filaments were exposed to 75 mM NaCl for 4 h (Fig. 5A and 5B). The maximal accumulations of trehalose and sucrose, about 0.48 mg/g dry weight (DW) and 2.40 mg/g DW respectively, also occurred under 75 mM NaCl stress, markedly about 3.4-fold and 2.9-fold increased compared with those of the control (0 mM NaCl), about 0.12 mg/g DW of trehalose and 0.54 mg/g DW of sucrose, respectively (Fig. 5C). However under 175 mM NaCl stress, both enzyme expressions and sugar contents were low, likely because of the extreme NaCl stress pressure to the filaments of *N. flagelliforme*. This limit in yield of trehalose was due to the side-hydrolysis reactions of maltooligosaccharides catalyzed by MTS and MTH [15]. However, the substrate specificity of MTH on this sidehydrolysis reaction has received little attention. The expression of MTS was very high at 150 mM NaCl stress, mostly caused by loading deviation.

The interesting results were derived when the rehydrated filaments of *N. flagelliforme* were exposed to various temperatures of 4°C, 10°C, 25°C (control), 37°C, and 42°C, respectively, in the air for 4 h. Under such treatment progress, the filaments suffered the temperature stress combined with the dehydration stress at the same time. The results showed that the expressions of NfMTS and NfMTH were significantly upregulated under high temperatures of 37°C and 42°C in the air, but significantly downregulated under low temperatures of 4°C and 10°C in the air for 4 h (Fig. 6A and 6B). The maximal upregulations of NfMTS and NfMTH happened when exposed to 42°C in the air for 4 h, with about 2.5-fold and 1.9-fold increase compared with those exposed to 25°C in the air for 4 h. Accordingly, the maximal accumulation of trehalose and sucrose was shown at 42°C in the air for 4 h, about 1.05 mg/g DW and 0.80 mg/g DW, respectively, which were nearly 2-fold and 6-fold higher than those at 25°C for 4 h (Fig. 6C). Small quantities of trehalose and sucrose were detected when the filaments were exposed to 4°C and 10°C in the air for 4 h.

Discussion

Continuing the work of cloning of *MTS* homologous genes of the gene cluster of *MTS-MTH-TreH* from *N. flagelliforme* by using degenerate primers [23], we successfully cloned another trehalose-synthesis gene, *MTH* of *N. flagelliforme*, in this work. The multiple sequence alignment of the deduced amino acid of NfMTH and other MTHs revealed that the conserved domains of NfMTH are highly conserved as those of the α-amylase family enzymes (underlined in Table 2), supporting the functional classification of NfMTH belonging to the α-amylase family [7, 12, 14]. Sequential reactions of recombinant NfMTS and recombinant NfMTH with the substrate maltohexaose (G6) demonstrated that the recombinant NfMTH has the function to release trehalose by cleaving the α-1,4-glucosidic linkage next to the α-1,1-linked terminal disaccharide of maltooligosyl trehalose (Fig. 3).

Trehalose was reported to have high water-holding activity and thus to preserve the integrity of biological membranes owing to its nonreducing α-1,1-linkage [3, 4, 6]. Therefore, it plays great roles in the protection and stabilization of biological structures such as cell membrane, protein, and nucleic acid under various stress conditions and significantly

enhances an organism's resistance [20]. A wide variety of organisms including yeast, desert plants, as well as some cyanobacterium species were reported to accumulate trehalose and sucrose under stress conditions [10, 11, 13, 20, 21]. Large amounts of trehalose or sucrose are required to work as "chemical chaperones" for the protection of the structure of proteins and membranes against denaturation and aggregation under dehydration [3]. In *Nostoc* species, few species accumulated trehalose and most accumulated sucrose as a compatible solute under salt and osmotic shock [19]. In *N. commune* under desiccation, both trehalose and sucrose accumulated [11, 24]. In *Anabaena* PCC 7120, very small quantities of trehalose (0.02–0.05%) and high quantities of sucrose (1–2%) were detected under dehydration [10]. In our study, the expressions of NfMTS and NfMTH were upregulated at the similar levels, and both trehalose and sucrose were obviously accumulated when *N. flagelliforme* was exposed to drought, salt, and temperature-drought stresses. Under drought stress, up to 0.4 mg/g DW of trehalose and about 1.0 mg/g DW of sucrose accumulated in *N. flagelliforme* [23]. However, under NaCl stress, as much as 2.5 mg/g DW of sucrose accumulated in that microbe, whereas only about 0.5 mg/g DW of trehalose accumulated (Fig. 5C). Since *N. flagelliforme* usually grows on dry desert steppes, bare lands, or hills in dry or semi-dry areas, it often experiences severe drought and high temperature stresses in nature and shows marked desiccation tolerance and extreme heat resistance. The trehalose and sucrose contents were detected under temperature-drought stress conditions by exposing the filaments to various temperatures in the air for 4 h. The results showed that higher trehalose accumulated than sucrose did under high temperature-drought stresses (Fig. 6C). Therefore, *N. flagelliforme* might conduct different strategies to adapt to different stresses. When sucrose accumulation predominated, it might be sucrose but not trehalose that is more likely to function as a chemical chaperone in the protection of macromolecules, such as under NaCl stress and dehydration stress. On the contrary, it might be trehalose that is more likely to work as a chemical chaperone in adaptation to conditions such as high temperature (higher than 37°C)-drought stress. Under low temperatures (lower than 10°C), the thin filaments of *N. flagelliforme* might become dormant immediately and the protein synthesis and other physiological metabolism stopped quickly. Therefore, little NfMTS, NfMTH, sucrose, and trehalose were detected (Fig. 6).

The trehalose metabolism in *Anabaena* PCC7120, *N. commune*, and many hyperthermophilic archaeum bacteria is controlled by the three enzymes MTH (TreZ), MTS (TreY), and TreH (trehalase), which are found as a gene cluster [1, 10, 24]. The genes of MTH, MTS, and TreH in *N. flagelliforme* were also confirmed to be sequentially located at a long DNA fragment and were a gene cluster under the same operon regulation (unpublished data). The TreH activity was reported to be inhibited under NaCl stress but not under desiccation stress [24]. Therefore, the content of trehalose might be determined by the sequential reactions of *MTS*, *MTH*, and *TreH* under different conditions. The control of trehalase activity might also play an important role in trehalose accumulation and adaptation in terrestrial cyanobacteria under stress conditions. In *Anabaena* PCC7120, a low level of trehalose could induce the expression of a cofactor of the chaperone *DnaK* gene, which is important for the dehydration tolerance of this species [10]. The extracellular polysaccharides on the thicker sheath surface of filaments were demonstrated to play a crucial role in desiccation tolerance for *N. commune* [22] and also for *N. flagelliforme* [8]. Therefore, the mechanisms of its extreme desiccation and heat tolerance most likely involve multiple protection processes and need further studies in our laboratory.

Acknowledgments

This work was supported by the Key Fundamental Project of Shanghai (03JC14057), Innovation Program of Shanghai Municipal Education Commission (05DZ29), Leading Academic Discipline Project of Shanghai Municipal Education Commission (No. J50401), and Leading Academic Discipline Project of Shanghai Normal University (No. DZL808).

REFERENCES

1. Allewalt, J. P., M. M. Bateson, N. P. Revsbech, K. Slack, and D. M. Ward. 2006. Effect of temperature and light on growth of and photosynthesis by *Synechococcus* isolates typical of those predominating in the octopus spring microbial mat community of Yellowstone National Park. *Appl. Environ. Microbiol.* **72:** 544–550.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
3. Crowe, J. H., J. F. Carpenter, and L. M. Crowe. 1998. The role of vitrification in anhydrobiosis. *Annu. Rev. Physiol.* **60:** 73–103.
4. Crowe, J. H., L. M. Crowe, and D. Chapman. 1984. Preservation of membranes in anhydrobiotic organisms: The role of trehalose. *Science* **223:** 701–703.
5. Dodds, W. K., D. A. Gudder, and D. Mollenhauer. 1995. The ecology of *Nostoc*. *J. Phycol.* **31:** 2–18.
6. Elbein, A. D., Y. T. Pan, I. Pastuszak, and D. Carroll. 2003. New insights on trehalose: A multifunctional molecule. *Glycobiology* **13:** 17R–27R.
7. Fang, T. Y., W. C. Tseng, T. Y. Shin, and M. Y. Wang. 2008. Identification of the essential catalytic residues and selectivity-related residues of maltooligosyltrehalose trehalohydrolase from the thermophilic archaeon *Sulfolobus solfataricus* ATCC 35092. *J. Agric. Food Chem.* **56:** 5628–5633.

8. Gao, K. 1998. Chinese studies on the edible blue-green alga, *Nostoc flagelliforme*: Review. *J. Appl. Phycol*. **10:** 37−49.
9. Gao, K. and D. Zou. 2001. Photosynthetic bicarbonate utilization by a terrestrial cyanobacterium, *Nostoc flagelliforme* (Cyanophyceae). *J. Phycol.* **37:** 768−771.
10. Higo, A., H. Katoh, K. Ohmori, M. Ikeuchi, and M. Ohmori. 2006. The role of a gene cluster for trehalose metabolism in dehydration tolerance of the filamentous cyanobacterium *Anabaena* sp. PCC 7120. *Microbiology* **152:** 979−987.
11. Hill, D. R., A. Peat, and M. Potts. 1994. Biochemistry and structure of the glycan secreted by desiccation-tolerant *Nostoc commune* (cyanobacteria). *Protoplasma* **182:** 126−148.
12. Kobayashi, K., M. Kato, Y. Miura, M. Kettoku, T. Komeda, and A. Iwamasu. 1996. Gene cloning and expression of new trehalose-producing enzymes from hyperthermophilic archaeum *Sulfolobus solfataricus* KM1. *Biosci. Biotech. Biochem*. **60:** 1882−1885.
13. Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56:** 658−666.
14. MacGregor, E. A., S. Janecek, and B. Svensson. 2001. Relationship of sequence and structure to specificity in the alpha-amylase family of enzymes. *Biochim. Biophys. Acta* **1546:** 1−20.
15. Mukai, K., A. Tabuchi, T. Nakada, T. Shibuya, H. Chaen, S. Fukuda, M. Kurimoto, and Y. Tsujisaka. 1997. Production of trehalose from starch by thermostable enzymes from *Sulfolobus acidocaldarius*. *Starch Staerke* **49:** 26−30.
16. Nakada, T., K. Maruta, K. Tsusaki, M. Kubota, H. Chaen, T. Sugimoto, M. Kurimoto, and Y. Tsujisaka. 1995. Purification and properties of a novel enzyme, maltooligosyl trehalose synthase, from *Arthrobacter* sp. Q36. *Biosci. Biotech. Biochem*. **59:** 2210−2214.
17. Pang, W. T., S. X. Wu, J. Yu, and Q. X. Wang. 2007. Determination of trehalose and sucrose contents in *Nostoc flagelliforme*. *J. Shanghai Normal University* (Natural Sciences Edition). **36:** 73−76.
18. Pramanik, M. H. R. and R. Imai. 2005. Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. *Plant Mol. Biol.* **58:** 751−762.
19. Reed, R. H., D. L. Richardson, S. R. C. Warr, and W. D. P. Stewart. 1984. Carbohydrate accumulation and osmotic stress in cyanobacteria. *J. Gen. Microbiol.* **130:** 1−4.
20. Schiraldi, C., I. Di. Lernia, and M. De. Rosa. 2002. Trehalose production: Exploiting novel approaches. *Trends Biotechnol.* **20:** 420−425.
21. Seo, J.-S., J. H. An, M.-Y. Baik, C. S. Park, J.-J. Cheong, T. W. Moon, K. H. Park, Y. D. Choi, and C. H. Kim. 2007. Molecular cloning and characterization of trehalose biosynthesis genes from hyperthermophilic archaebacterium *Metallosphaera hakonesis*. *J. Microbiol. Biotechnol.* **17:** 123−129.
22. Tamaru, Y., Y. Takani, T. Yoshida, and T. Sakamoto. 2005. Crucial role of extracellular polysaccharides in desiccation and freezing tolerance in the terrestrial cyanobacterium *Nostoc commune*. *Appl. Environ. Microbiol*. **71:** 7327−7333.
23. Wu, S. X., R. R. Shen, X. Zhang, and Q. X. Wang. 2010. Molecular cloning and characterization of maltooligosyltrehalose synthase gene from *Nostoc flagelliforme*. *J. Microbiol. Biotechnol.* **20:** 579−586.
24. Yoshida, T. and T. Sakamoto. 2009. Water-stress induced trehalose accumulation and control of trehalase in the cyanobacterium *Nostoc punctiforme* IAM M-15. *J. Gen. Appl. Microbiol*. **55:** 135−145.