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Molecular Cloning and Characterization of Maltooligosyltrehalose Synthase Gene from *Nostoc flagelliforme*

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A genomic DNA fragment encoding a putative maltooligosyltrehalose synthase (*NfMTS*) for trehalose biosynthesis was cloned by the degenerate primer-PCR from cyanobacterium *Nostoc flagelliforme*. The ORF of *NfMTS* was 2,799 bp in length and encoded 933 amino acid residues constituting a 106.6 kDa protein. The deduced amino acid sequence of *NfMTS* contained 4 regions highly conserved for MTSs. By expression of *NfMTS* in *E. coli*, it was demonstrated that the recombinant protein catalyzed the conversion of maltohexaose to maltooligosyl trehalose. The K_m of the recombinant enzyme for maltohexaose was 1.87 mM and the optimal temperature and pH of the recombinant enzyme was at 50°C and 7.0, respectively. The expression of MTS of *N. flagelliforme* was upregulated, and both trehalose and sucrose contents increased significantly in *N. flagelliforme* during drought stress. However, trehalose accumulated in small quantities (about 0.36 mg/g DW), whereas sucrose accumulated in high quantities (about 0.90 mg/g DW), indicating both trehalose and sucrose were involved in dehydration stress response in *N. flagelliforme* and sucrose might act as a chemical chaperone rather than trehalose did during dehydration stress.

Keywords: *Nostoc flagelliforme*, maltooligosyltrehalose synthase, degenerate primer, desiccation stress, cyanobacterium

Nostoc flagelliforme is a type of terrestrial and macroscopic cyanobacterium distributed in the northern and west-northern parts of China, and in Mongolia, Russia, Algeria, Czechoslovakia, Morocco, Somalia, France, Mexico, and U.S.A. [6, 8]. It contains 17 types of amino acids, including 8 essential ones for humans, which are much higher than those in a mushroom, *Lentinula edodes*. It also has anti-aging and antitumor activities and can enhance the body immune system [8]. Therefore, it has been used as a food delicacy and an herbal medicine ingredient by the Chinese for centuries. Many studies have been performed on its morphology, ecology, physiology, and cultivation in China [8, 9]. However, the field cultivation of this organism has not yet been successful. Owing to the extensive overexploration and the serious disturbances of natural habitats, the resource of *N. flagelliforme* is increasingly limiting and it has been a protected species in China.

In nature, *N. flagelliforme* usually grows on dry desert steppes, bare lands, or hills in dry or semi-dry areas, where it often experiences severe environmental changes, under which other terrestrial *Nostoc* species hardly survive. In those areas, the highest annual mean temperature is around 35°C in summer and the lowest is −17°C in winter. The soil surface temperature can rise up to as high as 66°C in summer and down to as low as −29°C in winter. The rainy season is only from June to August, responsible for about 70% of the yearly precipitation. The annual evaporation is 10 to 20 times of the rainfall. The gray-brown soil or red soil is very poor and alkaline because of much dissolved calcium [8].

N. flagelliforme showed marked desiccation tolerance and extreme heat resistance. 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]. The filaments of *N. flagelliforme* dried for a couple of years could recover metabolic activity within hours after rehydration [6, 9]. The cylindrical filament of *N. flagelliforme* is believed to have a large surface/mass ratio to make it absorb and lose water faster than other terrestrial *Nostoc* species [8]. However, immersion of the filaments for 24 h enhanced the leakage to 9% while resulting in 60% and 90% electrolyte release, respectively, in 3 days and 9 days. Therefore, drought

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is not simply an environmental stress factor but is physiologically and ecologically significant to *N. flagelliforme* [8]. The molecular mechanism of *N. flagelliforme* adapted to such drought conditions is still poorly understood.

Trehalose has been detected in this organism in our previous work [22]. Two glucose units in this nonreducing disaccharide are linked with an glycosidic bond and form a α-D-glucopyranosyl-[1,1]-α-D-glucopyranose, which was reported in a variety of organisms including bacteria, fungi, yeast, invertebrates, and some plants to serve as a protective agent and/or carbohydrate reserves under stress conditions such as drought, heat, salinity, and oxidative stress [7]. Trehalose is known to have high water-holding activity and thus to preserve the integrity of biological membranes [4, 5, 7]. 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 the organism's resistance [28]. Until now, five pathways for trehalose biosynthesis have been identified. In *E. coli*, yeast, and plants, trehalose biosynthesis is accomplished *via* trehalose-6-phosphate (T6P) synthase converting UDP-glucose and glucose-6-phosphate into T6P that is further dephosphorylated to trehalose by T6P phosphatase [7, 18]. In the fungus *Grifola frondosa*, trehalose phosphorylase (TreP) was discovered to catalyze D-glucose and α-D-glucose-1-phosphate to produce trehalose [26]. In the TreS pathway, the trehalose synthase (TreS) has strict substrate specificity for maltose and produces trehalose from maltose in one step by intramolecular transglucosylation, such as found in *Mycobacterium smegmatis* [21], *Pimelobacter* sp. R48, and *Thermus aquaticus* ATCC 33923 [28]. A group of thermophilic archaebacteria and several mesophilic eubacteria such as *Metallosphaera hakonesis* [29], *Sulfolobus*, *Arthrobacter*, *Rhizobium*, and *Brevibacterium* (details in the review of Schiraldi *et al.* [28]) produce trehalose 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 [20, 28]. MTS functions with a variety of maltooligosaccharides having numbers of glucose molecules in the range from 4 to 7 [20]. Several species of genus *Nostoc* accumulated trehalose as compatible solute under salt and osmotic shock [11, 25]. *Anabaena* PCC 7120, a close species related to genus *Nostoc*, was also reported to accumulate trehalose under desiccation and salinity stress [10, 24]. It has been proven that trehalose synthesized *via* the MTS–MTH enzymatic system and the gene cluster consisting of MTH, MTS, and treH (encoding trehalase) was upregulated under dehydration stress in *Anabaena* PCC 7120 [10] and *Synechococcus* sp. JA-3-3Ab [1]. Therefore, a crucial role of trehalose-metabolizing enzymes in dehydration tolerance was expected in *N. flagelliforme*. In this study, we cloned a genomic DNA fragment encoding a putative maltooligosyltrehalose synthase (NfMTS) for trehalose biosynthesis from *N. flagelliforme* by degenerate primer-PCR, and the characteristics of the recombinant NfMTS were studied. NfMTS expression level and trehalose and sucrose contents were detected during the dehydration process in *N. flagelliforme*. The possible protection mechanism of trehalose in *N. flagelliforme* is discussed.

Materials and Methods

Algal Materials and Sample Preparation

The filament of *N. flagillifome* was collected from the Inner Mongolia Autonomous Region of China in the summer of 2004 and 2008. The filaments were stored in the desiccation cabinet at room temperature before being used for experiments. When used for experiments, the filaments were washed with double-distilled water 3 times and rehydrated for 24 h before 0.2–0.5 g of the filaments was weighted for DNA and protein extraction or for drought stress treatments by exposure in the air at room temperature for 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h.

Total DNA Extraction

A 0.2 g sample of rehydrated (24 h) filament of *N. flagillifome* was ground in liquid nitrogen to powder. The powder was solved in 1 ml of extraction buffer [100 mM Tris-HCl, 20 mM EDTA, 2% lauroyl sarcosine (w/v), 42% urea (w/v)]. The mixture was extracted with 1 ml of phenol and chloroform–isoamyl alcohol (24:1 in volume), respectively, and centrifuged at the maximum speed at 4°C for 20 min to get the aqueous phase. Total DNA was precipitated with an equal volume of isopropanol and 1/10 volume of NaAC. The DNA pellet was dried in air and dissolved in Tris-EDTA buffer (pH 8.0) and stored at −20°C before PCR amplification.

Cloning of *NfMTS* Gene

According to the amino acid sequences of *MTS* genes of *N. punctiforme* PCC 73102 (CP001037.1; GI: 186463002) and *Anabaena* PCC 7120 (BA000019.2; GI:47118302), the alpha-amylase gene of *Anabaena variabilis* ATCC 29413 (CP000117.1; GI:75699950), and the codon bias of cyanobacteria, degenerate primers were designed as follows for PCR to clone the coding region of *NfMTS* from the genomic DNA of *N. flagillifome*: Upstream primer 5'-atgcgaattcctammgcwac-3'; downstream primer 5'-aagcgrggsgcgatsgcgat-3'. The touchdown PCR amplification was performed in 50-μl reaction mixtures with hotstart *Taq* polymerase (Invitrogen). The annealing temperature (30 s) was from 65°C to 55°C for the first 10 cycles, and in each cycle the annealing temperature decreased with 1°C followed by 25 more cycles with constant annealing reaction at 55°C. In each reaction cycle, the denaturation was at 94°C for 30 s and the elongation was at 72°C for 4 min. All assays began with a denaturation temperature of 95°C for 4 min and ended with an elongation time of 20 min. The products of PCR amplification were assayed by electrophoresis in agarose gel and purified with Wizard PCR Prep kits (Promega).

The purified PCR products were ligased into pGEM Easy-T (Promega) vector and then transformed into *E. coli* strain DH5α. The correct transformants were determined by restriction enzyme assay and PCR and subjected to sequencing respectively. To construct plasmid for expression of recombinant *NfMTS* in *E. coli* strain BL21, the clone was ligated into plasmid pET32b (Novagen) *via* *Bam*HI and *Sal*I sites to form the expression plasmid pET32b-*MTS*.

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

One hundred ml of broth of *E. coli* strain BL21 was taken after 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) induction at 37°C for 6 h and centrifuged at 6,000 rpm for 10 min to collect cells. The cell pellet was washed in 1 ml of Tris-HCl (pH 7.4) and resuspended in 30 ml of binding buffer (10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 500 mM NaCl, 30 mM imidazole, pH 7.4) and sonicated for lysis. The suspension was centrifuged at 10,000 ×*g* for 10 min to clarify the enzyme solution. The recombinant protein was expressed mostly as an insoluble one and was analyzed with SDS-PAGE. The recombinant protein products were purified by Ni^{2+}-NTA-agarose affinity chromatography with AKTA Purifier following the manufacturer's manual (GE Healthcare Co. Ltd, U.K.). Protein was quantified spectrophotometrically as Bradford [2] with bovine serum albumin (BSA) as the standard.

MTS Activity Assay

MTS activity was measured by incubating 7 μg of purified recombinant enzymes and 2.5 mg of maltohexaose (Supelco) in 500 μl of 50 mM phosphate-citric acid buffer (0.2 M Na_3PO_4, 0.1 M citric acid, pH 7.0) at 50°C for 30 min. MTSase converts the reducing terminal α-1,4-linked residue of maltohexaose into α-1,1 linkage. The reaction was terminated by heating at 100°C for 10 min. After cooling, the solution was adjusted to pH 4.2 and 30 μl of glucoamylase Dextrozyme DX (Novozymes) was added to incubate at 60°C for 24 h, which only hydrolyzes α-1,4-linked glucose from maltotetraosyl trehalose to produce glucose and trehalose [3]. The reducing sugar and trehalose were analyzed by high-performance liquid chromatography (HPLC), described below. One unit of the MTS activity was defined as the amount of enzyme required to convert 1 μmol maltohexaose into maltotetraosyl trehalose in 1 min under the above conditions. The K_m of MTSase was analyzed according to the method of Lineweaver and Burk [17].

Crude Protein Preparation and MTS Activity Assay of Algae

The filament of *N. flagelliforme* was ground in liquid nitrogen. Total soluble proteins were extracted by sonication of the ground samples in Medium A (5 mM sodium phosphate, pH 7.5, 10 mM $MgCl_2$, 10 mM NaCl, 25% glycerol, 10 mM HEPES) at 4°C for 15 min. Each extract was centrifuged at 13,000 ×*g* for 15 min and the supernatant was collected. About 10 μg of total proteins was added into a 300-μl reaction mixture to conduct MTS activity assay as previously mentioned.

Trehalose Extraction and Determination in Algae

After drought treatment, 0.2 g of the filament of *N. flagelliforme* was ground with 1.5 ml of 100% ethanol and held at 4°C for 4 h. The mixture was centrifuged at 13,000 ×*g* for 10 min and the supernatant was volatilized to evaporate the ethanol at room temperature. The residues were dissolved in 100 μl of 100% ethanol for HPLC detection (Waters, 600 Controller) equipped with a ZORBAX-NH_2 column (4.6 mm×250 mm, pore size 5 μm; Agilent, U.S.A.). The oligosaccharides loaded onto the column were eluted with constant acetonitrile-water eluent [75:25 (v/v)] at 1.0 ml/min flow velocity over 20 min and the column temperature was 30°C. The eluted oligosaccharide fractions were detected by the evaporative light scattering detector (Polymer Laboratories, PL-ELS 2100). The trehalose peak (in standard or in sugar extracts) was also confirmed by digestion with trehalase (Sigma), which converts trehalose to glucose [23].

Western Blotting

Five μg of the crude soluble protein samples was separated by using SDS polyacrylamide gel electrophoresis (SDS-PAGE) with 6% seperating gels and 5% collecting gels according to the standard protocol. Proteins were transferred to the polyvinylidene fluoride (PVDF; Millipore, U.S.A.) membrane and MTS protein was detected by polyclonal anti-MTS antibodies. Chemiluminescence was detected using the Amersham Enhanced Chemiluminescent (ECL) Plus Western Blotting Detection System (GE Healthcare Co. Ltd., U.K.) on X-ray film. The optical density from Western blot was conducted by using the Tannon Gel Image System (Tannon, Shanghai, China).

RESULTS

Cloning of *NfMTS* Gene from *N. flagillifome*

Since trehalose was detected in *N. flagelliforme* previously in our laboratory, the biosynthesis of trehalose was assumed in this drought-adapted and heat-resistant organism. *N. flagelliforme* has a close relationship with *N. punctiforme* and *Anabaena* sp., which were reported to have the MTS-MTH trehalose biosynthesis pathway. Therefore, we designed degenerate primers according to the amino acid sequences of *MTS* of the above species and considering the codon bias in cyanobacteria. A genomic DNA fragment coding the ORF region of *MTS* (*NfMTS*) was cloned from *N.*

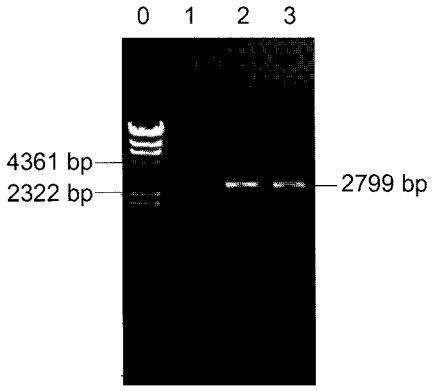

Fig. 1. The electrophoresis assay for identification of *NfMTS* cloned from genomic DNA of *N. flagelliforme* by the touchdown PCR method.

Lane 0: DNA molecular marker. Lane 1: Negative control, without templates in the PCR. Lanes 2 and 3: PCR product, about 2,799 bp, from genomic DNA of *N. flagelliforme*.

Table 1. 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	MTSs	** : .***	*********	* .** : **	: : :***
	NfMTS.	86 QDIVPNH	326 GLRI**D**HIDG	353 YITV**E**KIL	595 TTATH**D**
	NpMTS	86 QDIVPNH	326 GLRI**D**HIDG	353 YITV**E**KIL	595 TTATH**D**
	NpMTS 73102	86 QDIVPNH	326 GLRIDHIDG	353 YITV**E**KIL	595 TTATHD
	A7120MTS	86 QDIVPNH	326 GLRI**D**HIDG	353 YITV**E**KIL	595ATATH**D**
	AvAmyl	86 QDIVPNH	326 GLRI**D**HIDG	353 YVTV**E**KIL	595ATATH**D**
	SynJA3MTS	86 QDIVPNH	326 GLRI**D**HIDG	353 YIVV**E**KIL	595 ASSTH**D**
	SfMTS	87 QDMVPNH	330 GLRI**D**HIDG	357 YTVV**E**KIL	599 TTSTH**D**
	SsMTS#	84 QDIVPNH	224 GYR**I**D**H**IDG	251 I I IV**E**KIL	438 ATSTH**D**

Row A: The generalized sequences of four conserved regions [19]. X: Hydrophobic residue; B: hydrophilic residue; O: Gly or Ala residue; Z: residue important for specificity. Row B: Trehalosyl dextrin-forming enzymes (MTSs). The identical residues among these MTSs are marked by asterisks. The three proposed catalytic residues of Asp, Glu, and Asp in regions 2, 3, and 4 respectively, are shown in boldface. The residues existing in the active site of NfMTS are underlined. MTSs: Maltooligosyltrehalose synthases; NfMTS: MTS of *N. flagelliforme*; NpMTS: MTS of *N. punctiforme*; NpMTS 73102: MTS of *N. punctiforme* 73102; A7120MTS: MTS of *Anabaena* PCC 7120; AvAmyl: alpha-amylase of *A. variabilis* ATCC 29413; SynJA3MTS: MTS of *Synechococcus* sp. JA-3-3Ab; SfMTS: MTS of *Syntrophobacter fumaroxidans*; SsMTS: MTS of *Sulfolobus solfataricus*.

flagelliforme by the touchdown PCR method using the degenerate primers (Fig. 1). The sequencing results showed that the ORF region of *NfMTS* to be 2,799 bp in length and encoded 933 amino acid residues constituting 106.6 kDa. *NfMTS* showed 92%, 89%, and 78% homology with the *MTS* genes from *N. punctiforme, N. punctiforme* PCC 73102, and *Anabaena* sp. PCC 7120, respectively, and also showed 78% overall identity with the catalytic region of the alpha-amylase gene of *A. variabilis* ATCC 29413 (data not shown). The nucleotide sequence of the *NfMTS* gene has been deposited to the GenBank database under Accession No. EF433294.

The deduced amino acid sequence of NfMTS was compared with the homologous enzymes from *N. punctiforme* PCC 73102 (NpMTS), *A. variabilis* ATCC 29413 (AvAmyl), *Anabaena* sp. PCC 7120 (A7120MTS), *Synechococcus* sp. JA-3-3Ab (SynJA3MTS), *Synechococcus* sp. JA-2-3B'a (2-13) (SynJA2MTS2), *Syntrophobacter fumaroxidans* (SfMTS), and *Sulfolobus solfataricus* (SsMTS). NfMTS showed as high as 95% identity with NpMTS, 80% identiy with AvAmyl, 79% identity with A7120MTS, 56% identity with SynJA3MTS and SynJA2MTS2, and 49% identity with SfMTS. The multiple sequence alignment revealed four regions highly conserved in the active sites of MTSs (Table 1). Three proposed catalytic residues, Asp, Glu, and Asp in conserved regions 2, 3, and 4, respectively (shown in boldface in Table 1), and the residues existing in the active site of α-amylase family enzymes (underlined in

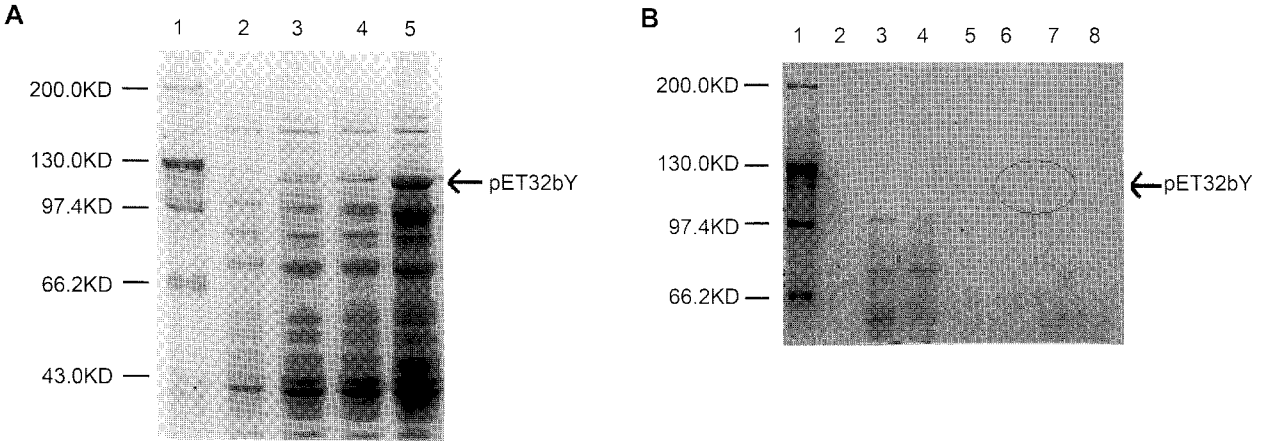

Fig. 2. Expression and purification of the recombinant NfMTS proteins in *E. coli* strain BL21.
The arrow indicates recombinant NfMTS. **A**. The recombinant NfMTS was demonstrated to be mainly insoluble, assayed by SDS–PAGE. Lane 1, Molecular mass standards; Lane 2, total protein of *E. coli* without induction; Lanes 3 and 4, soluble protein of *E. coli* induced by 1 mM IPTG at 37°C for 1 h and 6 h, respectively; Lane 5, insoluble protein of *E. coli* induced by 1 mM IPTG at 37°C for 6 h. **B**. SDS–PAGE analysis of purified MTSase from *E. coli*. Lane 1, Molecular mass standards; Lanes 2–8, proteins eluted by AKTA purifier with different elution time; Lanes 5–7, purified proteins used for enzyme assay. The separation gel of SDS–PAGE was with 6% polyacrylamide and stained with Coomassie Brilliant Blue.

Table 1), were also highly conserved in the amino acid sequences of NfMTS.

Purification and Activity of Recombinant NfMTS Enzyme

The expression of recombinant NfMTS was induced by 1 mM IPTG from the vector pET32bY 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 125 kDa, including the hexahistidine domain from the pET32bY vector (Fig. 2A). This value is in agreement with the value predicted from its deduced amino acid sequence.

The soluble protein product of pET32bY was purified by Ni^{2+}-NTA-agarose affinity chromatography (Fig. 2B). The purified recombinant NfMTS was quantified and incubated with maltohexaose, a reaction in which MTS exerted a glucosyltransferase activity at the reducing end of maltohexaose (G6) to convert the first α-1,4-glycosidic linkage into α-1,1 linkage, producing an intermediate maltotetraosyl trehalose (G6T). The reaction was stopped by boiling for 5 min and glucoamylase was added to hydrolyze the second α-1,4-glycosidic linkage of the intermediate to release glucose and one molecule of trehalose at 60°C for 24 h. The final reaction mixture was examined by HPLC (Fig. 3). The results showed that the recombinant enzyme could catalyze the conversion from maltooligosaccharide to maltooligosyl trehalose and the latter was further hydrolyzed into trehalose and glucose by glucoamylase, confirming that this recombinant protein had the function of MTSase (Fig. 3C). The activity of recombinant NfMTS was about 0.1 U/mg protein.

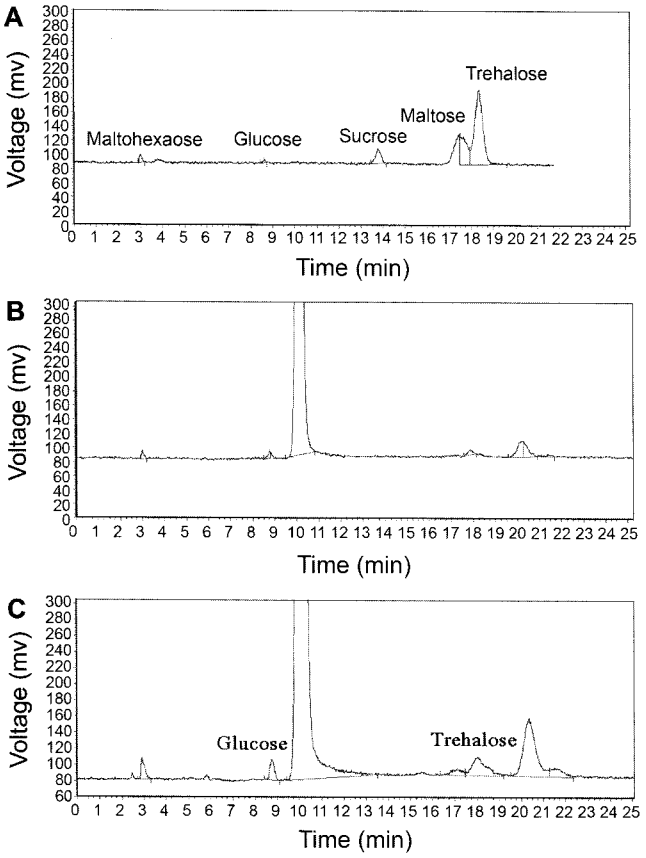

Fig. 3. Trehalose-producing activity of the recombinant NfMTS examined by HPLC.

Following the incubation of the purified recombinant NfMTS with (**C**)/ without (**B**) 5.05 mM maltohexaose at 50°C for 30 min in 50 mM phosphate buffers (pH 7.0), the enzyme mixture was heat-denatured and glucoamylase added for hydrolysis at 60°C for 30 min to produce glucose and trehalose. **A**. The profile of standards of maltohexaose, glucose, sucrose, maltose, and trehalose. **B**. The profile of the enzyme mixture without maltohexaose as the substrate. **C**. The profile of the enzyme mixture with maltohexaose as the substrate.

Characterization of Recombinant *NfMTS*

The optimum temperature of the recombinant NfMTS enzyme reaction for the trehalose production was about 50°C (Fig. 4A) and the optimum pH was around 7.0 (Fig. 4B).

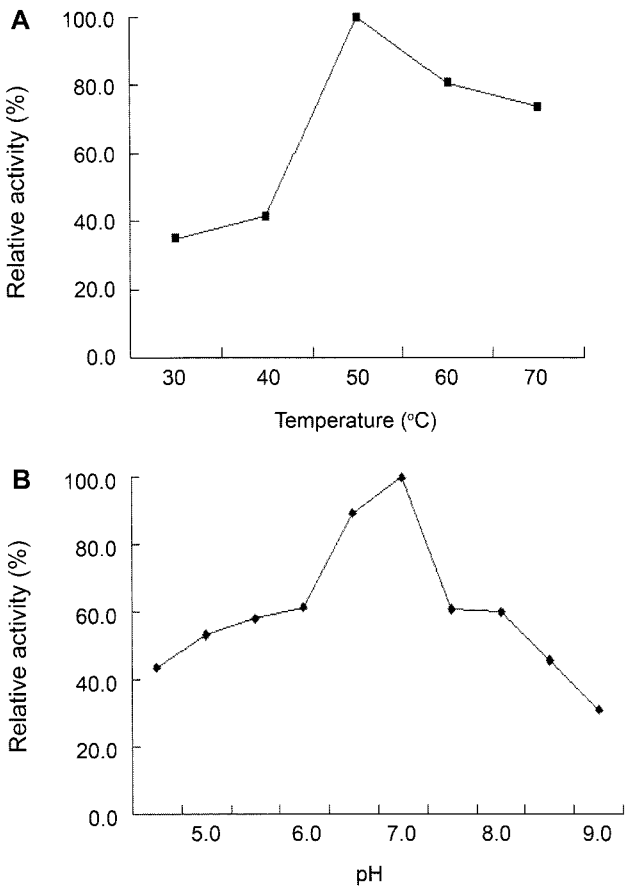

Fig. 4. Effects of temperature and pH on the activity of the MTSase.

The enzyme activity at various temperatures was studied at pH 5.5 and the enzyme activity at various pHs was studied at 50°C in 50 mM phosphate buffers for 30 min, using 5.05 mM maltohexaose as a substrate.

Table 2. Effects of metal ions on recombinant NfMTS activity.

Metal ions	Conc. (mM)	Relative activity (100%)
None	-	100
$CuSO_4$	1.0	54
$CaCl_2$	1.0	134
$HgCl_2$	1.0	42
$MnCl_2$	1.0	51
$MgCl_2$	1.0	66
$ZnSO_4$	1.0	122
EDTA	1.0	31

The enzyme was incubated in the presence of 1 mM of various metal ions at 50°C in 50 mM phosphate buffer (pH 7.0) for 30 min, using 5.05 mM maltohexaose as the substrate. The relative activity was expressed as a percentage of the enzyme activity in the absence of metal ions.

The effects of metal ions on the activity of recombinant NfMTS were determined in the presence of 1 mM of the metals under optimum reaction conditions (Table 2). The results showed that the enzyme activity was moderately inhibited by Mg^{2+}, Cu^{2+}, and Mn^{2+}, and extremely inhibited by EDTA, whereas Ca^{2+} and Zn^{2+} promoted the enzyme activity.

The K_m of the recombinant NfMTS for maltohexaose was calculated from Lineweaver–Burk plots and it was 1.87 mM, which was lower than MTSs found in *Arthrobacter* sp. Q36, *S. acidocaldarius*, *S. solfataricus* KM1, and *S. shibatae*, indicating NfMTS has high affinity to the substrate, maltohexaose.

Trehalose Accumulation and NfMTS Expression During Drought Stress

The change in the wet weight of filaments of *N. flagelliforme* during dehydration was determined. The wet weight decreased linearly during the first 8 h of dehydration and remained constant thereafter, at least up to 24 h. Therefore, we measured the contents of trehalose and sucrose and the expression level of NfMTS of *N. flagelliforme* during the dehydration process up to 24 h (Fig. 5). In the filaments of *N. flagelliforme* rewatered for 24 h, the trehalose level and sucrose was about 0.07 mg/g DW (dry weight) and 0.30 mg/g DW, respectively. Then, the filaments began to dry in the air at room temperature. The trehalose contents increased gradually to the maximum at 0.36±0.05 mg/g DW within 8 h during dehydration and remained constant thereafter, although the amount was small (corresponding to 0.03%–0.04% of dry weight) (Fig. 5A). Sucrose accumulated more significantly than trehalose did during the dehydration process, nearly three times to the maximum of 0.90±0.09 mg/g DW within 2 h, and decreased gradually thereafter (Fig. 5A).

Detected by the specific antibody, NfMTS protein contents increased rapidly to about 64.3% within 1 h and then gradually to the maximum, about 91.9% at 4 h during

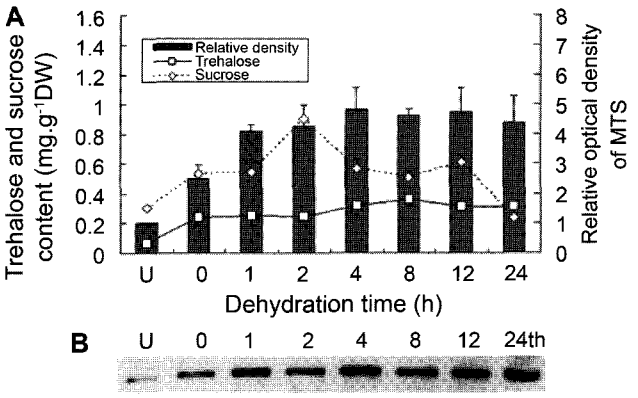

Fig. 5. Expression of NfMTS and accumulation of trehalose in filaments of during drought stress.

The filaments were dried in air at room temperature for 24 h. Trehalose and sucrose contents (**A**) and NfMTS expression level (**B**) were detected at the indicated time during the dehydration process. Ten μg of crude protein was used for the Western blotting to detect the NfMTS expression level by using specific MTS antibody. Experiments were done in at least triplicates.

dehydration (Fig. 5B). After 4 h of dehydration, the accumulation of NfMTS protein remained constant (Fig. 5B).

In dry samples of *N. flagelliforme* filaments, the contents of trehalose, sucrose, and NfMTS protein were much lower than those in the filaments after dehydration for 24 h (Fig. 5A and 5B). This might be due to the degradation of carbohydrates and proteins during the storage of the filaments.

Discussion

It was reported that in *Bradyrhizobium*, there were three enzymes for trehalose synthesis [30]. In this work, attempts were made to clone the *TPS/TPP* homologous genes from *N. flagelliforme*, but failed (data not shown). Only the *MTS*, *MTH*, and *treH* homologous genes were cloned from *N. flagelliforme* by using degenerate primers, and three genes were demonstrated to be in a gene cluster (unpublished data). However, the low homology at the beginning part of the coding region of *MTH* and at the end part of the coding region of the *treH* gene between *N. flagelliforme* and other species made the cloning of these two genes difficult and their characteristics are still being studied in our laboratory (unpublished data).

The multiple sequence alignment revealed that the conserved domains of NfMTS (Table 1) are common to all α-amylolytic enzymes such as α-amylases, pullulanases, cyclomaltodextrin glucanotransferases, and debranching enzymes, constituting substrate-binding sites of the starch hydrolysis enzymes [12–14]. The amino acid residues in the active site of NfMTS and α-amylase family enzymes (underlined in Table 1) are highly conserved, supporting the functional classification of NfMTS to the α-amylase

family [16, 19]. The recombinant NfMTS was demonstrated to have the function of maltooligosyltrehalose synthase. Furthermore, the NfMTS showed high similarity with MTSs of other species in genera *Nostoc* and *Anabaena*, indicating that its trehalose biosynthesis pathway might be similar to those found in prokaryotic bacteria such as *Nostoc* and *Anabaena* but not similar to those in *E. coli*, yeast, and higher plants.

The optimum pH value and temperature of the enzyme is often related to the species' living condition [28]. The optimum pH of NfMTS was 7.0, similar to those found in prokaryotic bacteria except species of *Sulfolobus*, the thermoacidophilic archaebacterium. The optimum temperature of NfMTS was 50°C, higher than those of common bacteria except *Thermus aquaticus*, a thermophilic species. Ca^{2+} could improve the activity of the recombinant NfMTS, indicating the adaptation of *N. flagelliforme* to high-temperature environments in the summer and to the soil condition in its habitat.

In *Nostoc* species, few species accumulated trehalose and most accumulated sucrose as a compatible solute under salt and osmotic shock [25]. In *N. commune* under desiccation, both trehalose and sucrose accumulated [11]. 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]. Yeast cells required at least 2–3% DW of trehalose under drought stress [27]. Most plants accumulated sucrose in large quantities under desiccation stress [18]. Therefore, very large amounts of trehalose and sucrose are required for working as "chemical chaperones," which is important in protecting the structure of proteins and membranes against denaturation and aggregation under dehydration, to confer a high drought stress tolerance to the organism [4]. In this study, trehalose did not accumulate much (about 0.04% DW) in *N. flagelliforme*, whereas sucrose accumulated markedly (about 1% DW) upon dehydration. Therefore, in *N. flagelliforme*, sucrose but not trehalose would be more likely to function as a chemical chaperone in protection of macromolecules under desiccation stress.

The accumulation of NfMTS protein in *N. flagelliforme* increased 64.3% within 1 h upon dehydration, but the accumulation of trehalose occurred gradually and was in small quantities during the dehydration process (Fig. 5). In our study, the genes of *MTH*, *MTS*, and *treH* for trehalose metabolism in *N. flagelliforme* was just confirmed to be sequentially located at a long DNA fragment and might be a gene cluster under the same operon regulation (unpublished data). The low trehalose content under dehydration might be the sequential reaction result of *MTS*, *MTH*, and *treH*. In *Anabaena* PCC 7120, 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]. Whether a similar function of trehalose or its derivatives is in *N. flagelliforme* under drought resistance and heat tolerance needs further study.

The filament of *N. flagelliforme* is usually covered with sheath, and it was reported that the thicker the sheath the higher the survival probability [8]. Our other study also demonstrated that the recombinant superoxide dismutase (SOD) gene of *N. flagelliforme* in *E. coli* showed higher stability than those of other *Nostoc* species under high temperature, salinity, and high pH stresses (unpublished). Therefore, there might be multiple adaptation mechanisms for *N. flagelliforme* to respond to salinity, drought, and temperature stresses in nature.

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