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Changes in the Amount of Cellular Trehalose, the Activity of Maltooligosyl Trehalose Hydrolase, and the Expression of Its Gene in Response to Salt Stress in the Cyanobacterium *Spirulina platensis*

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The amount of trehalose in cells of the cyanobacterium *Spirulina* (*Arthrospira*) *platensis* increased rapidly when a high concentration of NaCl was added to the culture medium. Inhibition of sodium ion transport by amiloride and monensin significantly decreased the amount of cellular trehalose, suggesting that the influx of sodium ions into the cells is coupled with the accumulation of trehalose. The amount of maltooligosyl trehalose hydrolase (Mth) which produces trehalose from maltooligosyl trehalose increased gradually after the increase in cellular trehalose. The gene for Mth was cloned and identified by Southern blot analysis. Real time RT-PCR analysis revealed that the expression of *mth* was enhanced by the addition of NaCl to the culture medium. It was concluded that both catalytic activity of Mth and the synthesis of Mth protein were enhanced by the addition of NaCl to the cells.

Key words: *Spirulina platensis*, trehalose, maltooligosyl trehalose hydrolase, salt stress

Cyanobacteria survive at various salt concentrations in habitats ranging from freshwater to seawater and, in extreme cases, even salt lakes (12, 18, 19, 20, 21). This tolerance for salt differs according to where they live. Each cyanobacterium contains a low molecular substance such as trehalose, sucrose, glucosyl glycerol or glycine-betaine, as a compatible solute to protect membranes and cellular proteins against salt stress (7, 17). Trehalose is a non-reducing disaccharide in which two glucose units are connected by an α,α-1,1-glycosidic linkage, and is distributed widely in various organisms from bacteria to higher plants. Recently, we have found that the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 accumulates trehalose and sucrose when exposed to desiccating conditions (9). The expression of genes in response to draught stress was determined using a DNA microarray of this cyanobacterium (9, 11, 25), and up-regulation of genes related to trehalose metabolism has been detected (9, 11).

The filamentous, gliding cyanobacterium *Spirulina platensis* is used as a health supplement and a source of β-carotene, and its cellular pigment, phycocyanin, is a natural coloring agent (4). This cyanobacterium tolerates salt stress up to 750 mM NaCl, about 1.5-fold the concentration in sea water (8, 22, 23, 26). Accordingly, it can survive in lakes with high salt concentrations (12). It has been reported that *Spirulina* accumulates trehalose and glucosyl glyceride when exposed to high salt concentrations (23). However, the molecular mechanism responsible for the accumulation of these compatible solutes has not yet been determined.

Here we have determined the change in the amount of cellular trehalose together with the change in the activity of maltooligosyl trehalose hydrolase (Mth) which forms trehalose from maltooligosyl trehalose, in *Spirulina* cells exposed to salt at concentrations of up to 750 mM. The increase in cellular trehalose with the addition of NaCl depended upon the increase in both the catalytic activity and the amount of enzyme synthesized. The gene for Mth was cloned and its expression in response to salt stress was also determined.

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Materials and Methods

Cell culture

Spirulina (*Arthrospira*) *platensis* strain M-135 from the Institute of Applied Microbiology, which is now maintained in the culture collection of the National Institute of Environmental Sciences as strain NIES-39, was used. The cells were grown in SOT medium (15), containing 200 mM sodium bicarbonate at 25°C with bubbling of air and illuminated with fluorescent lamps at an intensity of 30 μmol photons m^{-2} sec^{-1}. The *Spirulina* cells were incubated with 1/10-diluted SOT medium for 2 weeks before experiments.

Determination of cellular trehalose

The amount of trehalose in the cells was determined as described previously (9). The *S. platensis* cells were harvested by filtration with a glass fiber filter and trehalose in the cells was extracted with 80% ethanol for 3 h at 65°C and centrifuged. Extraction was repeated twice, and the supernatants were combined and dried by evaporation. The residue was redissolved in 0.5 mL of water and then centrifuged for five min.

The amount of trehalose in the supernatant was determined using trehalase (Sigma-Aldrich, St. Louis, MO, USA). The supernatant (100 μL) was mixed with a reaction mixture (100 μL) containing 100 mM 2-morpholinoethanesulfonic acid/KOH (pH 6.0) and 5×10^{-3} U mL^{-1} trehalase (Sigma-Aldrich), and the liberated glucose was measured using a glucose test kit (Wako, Osaka, Japan). The amount of trehalose was expressed in terms of μg mg^{-1} chlorophyll. The absorbance of chlorophyll extracted by methanol was measured

at 665 nm and the chlorophyll concentration was calculated from the equation, 1 A_{665} unit=13.42 μg chlorophyll mL^{-1} (13).

Enzyme assays

The activity of maltooligosyl trehalose hydrolase (Mth) was determined as described previously (9). The cells on the glass fiber filter were suspended in a disruption buffer [20 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)/NaOH (pH 7.0), 5 mM 6-amino-n-caproic acid, 1 mM benzamidine HCl]. The cells were broken using a bead beater (Biospec Products, Bartlesville, OK, USA) in the presence of 1 mL of zircon beads. Cell debris was removed by centrifugation at 10,000×g for 5 min. The supernatant was centrifuged at 100,000×g for 30 min, and desalted on an NAP-10 column (GE Healthcare Bio-science, Piscataway, NJ, USA) equilibrated with the same buffer. The resulting supernatants, referred to as crude cell extracts, were used in enzyme assays.

The enzyme activity was measured by incubating cell extracts in 200 μL of 20 mM HEPES/NaOH buffer (pH 7.0) containing 1% maltoheptaose. The amount of trehalose formed from maltoheptaose was measured using trehalase (Sigma-Aldrich) and a glucose test kit (Wako) as reported previously (9). Protein concentrations were determined using the Bio-Rad Protein Assay, based on the method of Bradford (2).

Cloning of the mth *gene*

Genomic DNA was isolated from *S. platensis* as described previously (24). PCR was carried out with genomic DNA using the degenerate primers mthF3 (5'-TGGWSNGAYGAYTTYCAYCA-3') and mthR1.2 (5'-CCNAYYTGRTCRTGRTTYTG-3'), designed based on conserved regions of maltooligosyl trehalose hydrolase in cyanobacteria. The PCR product was cloned into the *Eco*RV site of pBluescript II SK+ (Stratagene, La Jolla, CA, USA) and sequenced.

Southern blot analysis

Three micrograms of genomic DNA was digested with *Acc*I and *Hin*cII. The DNA fragments were resolved on a 0.7% agarose gel and transferred to a nylon membrane (Hybond-N+; GE Healthcare Bio-Science). A digoxigenin-labeled DNA probe for the *mth* gene was prepared by PCR with the primer pair M13-M (5'-GGGTTTTC-CCAGTCACGAC-3') and M13-RV (5'-TTATGCTTCCGGCTCG-TATGTTGTG-3') using the plasmid bearing a part of the *mth* gene as the template. Hybridization was carried out in hybridization buffer [0.5 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, and 7% SDS] at 65°C for 15 h and then the membrane was washed in wash buffer (40 mM Na_2HPO_4 (pH 7.2) and 1% SDS) at 65°C (3). Detection of hybridized probes was carried out as described previously (5).

Real-time RT-PCR analysis

Real-time quantitative RT-PCR analysis was carried out according to Ehira and Ohmori (6). Total RNA was extracted essentially according to Mohamed and Jansson (14) and treated with DNase I (TaKaRa Bio, Ots, Shiga, Japan). Aliquots (1 μg) of total RNA were reverse transcribed with 1 pmol of a 16S rRNA-specific reverse primer, RTrrn16SR, and 0.05 pmol of a *mth* specific reverse primer, as described previously (6). Reactions were performed with a DNA Engine Opticon®2 System (Bio-Rad, Hercules, CA, USA) using SYBR®*Premix Ex Taq*™ (Takara Bio) in the presence of 250 nM primers under the following conditions: 1 min at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The fluorescence intensity of the SYBR Green dye was measured after each amplification step. Relative ratios were normalized with the value for 16S rRNA and represented as means of data from triplicate experiments. The primers used for quantitative PCR were as follows: RTrrn16sF (5'-CAGCTCGTGTCGTGAGATGT-3'), RTrrn16sR (5'-TTACGGGATTGGCTCAGACT-3'), RTmthF (5'-ACTGGAGATTTGACCGGGTAT-3'), RTmthR (5'-CAGCAT-ACCACAAATTGAGAGG-3').

Results and Discussion

Effect of NaCl on the cellular amount of trehalose and Mth activity

Fig. 1 shows the effect of different concentrations of NaCl on the amount of trehalose in *S. platensis* cells. Trehalose was undetectable when the cells were incubated with 1/10-diluted SOT culture medium containing about 20 mM NaCl for 2 weeks. The cellular trehalose content increased dependent upon the concentration of NaCl added to the culture medium. The amount accumulated was about 0.9 μmol mg^{-1} Chl, when 750 mM NaCl was added to the cells, and was unsaturated even at this high concentration.

Fig. 2 shows the change in the amount of cellular trehalose with the addition of NaCl. When 500 mM NaCl was added to the 1/10 diluted culture medium, the amount of trehalose in the cells increased rapidly within 10 min had reached 0.8 μmol mg^{-1} Chl after 1 h. Moreover, the amount decreased to one fourth 2 h after the transfer of the cells from a high (250 mM) to low (20 mM) NaCl-containing medium (Fig. 3). It was confirmed that *S. platensis* accumulated trehalose as a compatible solute under salt stress, and the response of the cellular trehalose level to the change in the surrounding NaCl concentration was very rapid. The activities to produce and/or degrade trehalose appeared quite sensitive to the concentration of NaCl in the culture medium. This rapid change in the cellular trehalose content suggests the trehalose level to be a signal for subsequent changes in cellular metabolism in response to salt stress.

Trehalose is formed from maltooligosyl trehalose by maltooligosyl trehalose hydrolase (Mth). Thus, the change in the cellular amount of Mth on the addition of NaCl to the cell suspension was determined. Fig. 4 shows that the total cellular activity of Mth increased with the addition of 500 mM NaCl, and peaked after 24 h (8 nmol trehalose formed min^{-1}

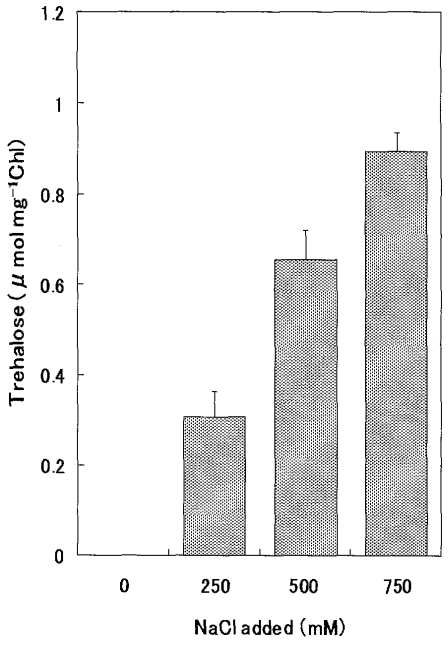

Fig. 1. Effects of NaCl concentrations on the cellular trehalose content of *S. platensis*. NaCl was added to a culture medium containing 20 mM NaCl. Trehalose was extracted from the cells after 1 h of incubation with different amounts of NaCl added.

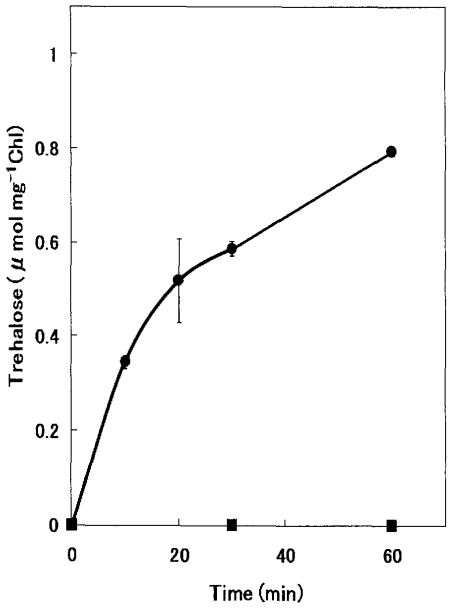

Fig. 2. Change in the cellular trehalose content of *S. platensis* after the addition of NaCl to the culture medium containing 20 mM NaCl. ●: 500 mM NaCl was added. ■: NaCl was not added.

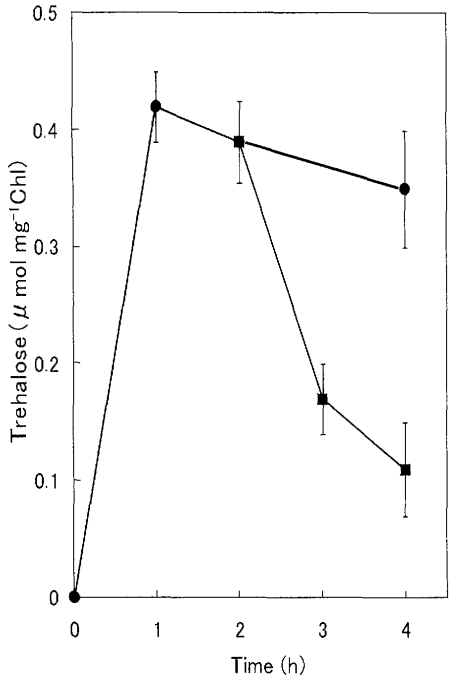

Fig. 3. Change in the trehalose content of *S. platensis* when cells were transferred from a high to low NaCl medium. ●: 250 mM NaCl was added to the culture medium containing 20 mM NaCl at time 0. ■: Cells were transferred to a low NaCl (20 mM) culture medium after 2 h of incubation with 250 mM NaCl added.

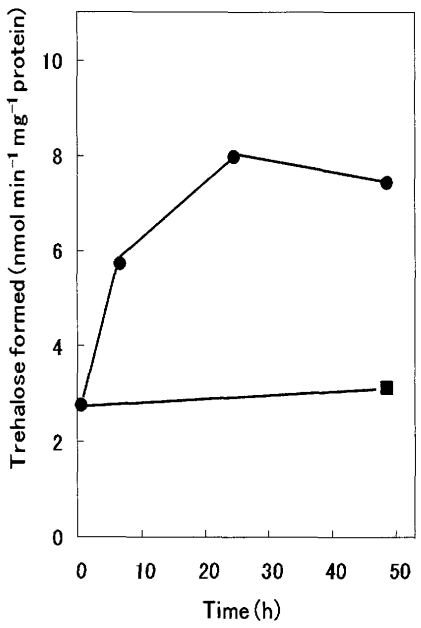

Fig. 4. Change in the activity of maltooligosyl trehalose hydrolase on the addition of 500 mM NaCl to *S. platensis* cell suspensions. ●: 500 mM NaCl was added at time 0. ■: Cells were incubated with a low NaCl (20 mM) culture medium from the start.

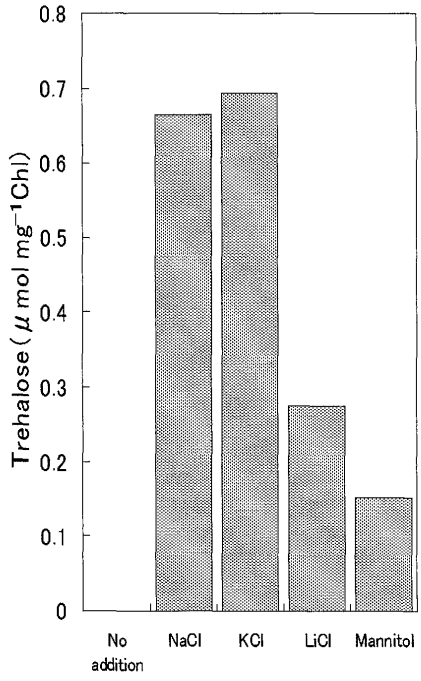

Fig. 5. Effect of cations and mannitol on the cellular trehalose content of *S. platensis*. Trehalose was extracted from the cells after 1 h of incubation with different cations (each 500 mM) and mannitol (500 mM).

mg^{-1} protein). In the control cell culture, where the NaCl concentration was low (20 mM), the enzyme activity remained at the initial level (2.6 nmol trehalose formed min^{-1} mg^{-1} protein). It is suggested that the increase in cellular trehalose on the addition of NaCl was supported by the rise in the amount of Mth in the cells. However, the increase in the total amount of Mth (Fig. 4) was rather gradual in comparison with the rapid accumulation of cellular trehalose (Fig. 3). Thus, the accumulation of cellular trehalose seems to depend upon the activation of Mth already present. It was also noted that no trehalose accumulated in the cells cultured with a low NaCl concentration, where the amount of Mth was constant though small (Fig. 4). It is considered that the level of activity of trehalase which breaks down trehalose to glucose is constant in the cells.

Effect of monovalent cations and inhibitors of Na^+ transport on the amount of cellular trehalose

Fig. 5 shows that the cellular amount of trehalose was

Fig. 6. Amino acid sequence alignment of a part of maltooligosyl trehalose hydrolase. The sequence of a part of the maltooligosyl trehalose hydrolase of *S. platensis* was reported in this study (spl, GenBank accession number AB282753). cya; Cyanobacteria Yellowstone A-prime, cyb; Cyanobacteria Yellowstone B-prime, npu; *Nostoc punctiforme* sp. PCC 73102, ana; *Anabaena* sp. PCC 7120, ava; *Anabaena variabilis* ATCC 29413. Arrows indicate the sequence used to design the degenerate primers.

Table 1. Effects of inhibitors for cation uptake on the NaCl-dependent accumulation of trehalose

Addition	Amount of trehalose[a] (μmol mg^{-1} Chl)	Relative amount (%)
NaCl[b]	0.575	100.0
NaCl[b]+Monensin[c]	0.289	50.3
NaCl[d]	0.391	100.0
NaCl[d]+Amiloride[c]	0.278	71.1

a; Values are means of duplicate determinations. b; 500 mM, c; 0.5 mM, d; 250 mM.

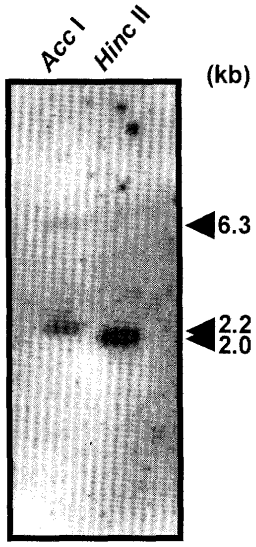

Fig. 7. Southern blot analysis of the *mth* gene. Genomic DNA (3 μg) of *S. platensis* was digested with *Acc*I and *Hinc*II. The sizes of detected bands are shown on the right of the panel. Details are described in Materials and Methods.

increased similarly by 500 mM Na^+ and 500 mM K^+, but only half as much by 500 mM Li. The addition of 500 mM mannitol was less effective than that of Na^+ or K^+. Even though the osmotic strength of the non-electrolytic mannitol is half that of the electrolytic NaCl or KCl, the effect of mannitol on cellular trehalose content was still less than half that of NaCl. It is suggested that the cells accumulated trehalose mainly via ionic stress rather than osmotic stress. In another cyanobacterium, *Anabaena* PCC 7120, we have found that NaCl but not sorbitol increased the cellular cAMP level, which suggests that salt stress and osmotic stress trigger quite different physiological responses (10).

Table 1 shows the effects of inhibitors of Na^+ transport on the NaCl-induced accumulation of trehalose in the cells. It was found that the addition of 0.5 mM amiloride, a sodium channel blocker, and 0.5 mM monensin, a cation ionophore, suppressed the accumulation by 70% and 50%, respectively. Thus the increase in the amount of cellular trehalose appears due to the enhanced influx of Na^+. It has been reported that ionic effects on the photosystems of a cyanobacterium, *Synechococcus* sp. PCC 7942, were caused by an influx of Na^+ ions through K^+/Na^+ channels (1). Previously, we have shown that the NaCl-dependent stimulation of respiration in *S. platensis* was inhibited by amiloride (16). The influx of Na^+, not the mere presence of Na^+ in the medium, seems coupled with the physiological effect of NaCl.

Cloning of the mth *gene*

To isolate the gene for Mth, part of *mth* was amplified using degenerate primers designed based on the sequence of a known *mth* (Fig. 6). The deduced amino acid sequence is similar to the Mth of *Anabaena variabilis* ATCC 29413 and *Anabaena* PCC 7120 showing 61% identity. Fig. 7 shows the result of a Southern blot analysis for the *mth* gene. The cloned region of the *mth* gene, in which there is a single *Acc*I site, was used as a probe, and double and single bands were detected for genomic DNA digested with *Acc*I and *Hinc*II, respectively. Thus the presence of *mth* in *S. platensis* cells was confirmed.

Change in the level of transcript of mth *on the addition of NaCl*

The change in expression of the *mth* gene in response to NaCl was determined using the real-time quantitative RT-PCR technique. Fig. 8 shows that the transcript of *mth* accumulated within 30 min when 500 mM NaCl was added to the cell suspension. The level of transcript peaked after 1 h and did not change further for 3 h. No significant change in the level of transcript was observed in the cells incubated in the 1/10 diluted SOT culture medium. These results suggest transcriptional regulation for the expression of *mth* in *S. platensis*, responding to the NaCl. The time lag observed between the amount of mRNA for *mth* (Fig. 8) and the amount of protein (Fig. 4) suggests a complicated sequential mechanism of translation and post-translational modifications in the course of protein synthesis. Recently, we have found that expression of the *mth* gene in *Anabaena* sp. PCC7120 was enhanced by draught stress (11) and that disruption of *mth* resulted in a decrease in cellular trehalose (9). The *mth* gene is probably involved in the general response to stress and trehalose could be a signal for a change in metabolism in order to adapt to new environments.

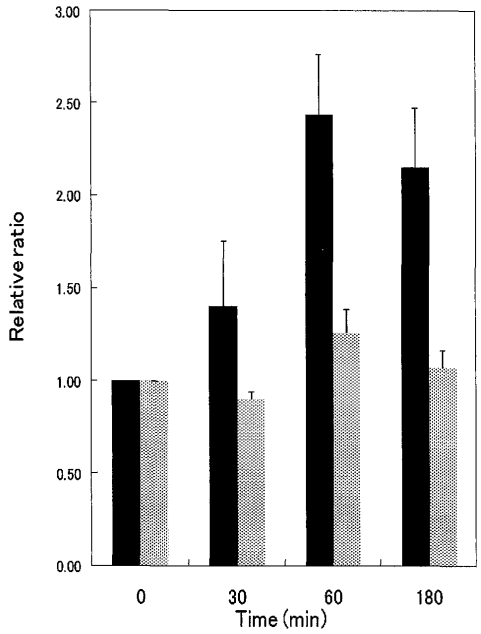

Fig. 8. Accumulation of the *mth* transcript under high NaCl conditions. Precultured cells were exposed to high salinity by adding 500 mM NaCl (Black bars). Cells were collected after 0, 0.5, 1, and 3 h, and the transcript level of *mth* was measured by real-time quantitative RT-PCR. Control experiments were carried out without the addition of NaCl (Shaded bars). Data are represented as the mean for triplicate experiments.

Conclusion

The addition of NaCl activated Mth, and at the same time triggered transcription of the *mth* gene. As a consequence of these biochemical and molecular biological events, the cellular amount of trehalose would be increased. Further investigation will be necessary to elucidate the entire mechanism of the NaCl-dependent accumulation of trehalose in the cell. Disruption of the *mth* gene, if this can be achieved, will provide new evidence concerning the role of Mth in the stress-responsive accumulation of trehalose in *Spirulina*.

Acknowledgements

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