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Effect of Trehalose Accumulation on the Intrinsic and Acquired Thermotolerance in a Natural Isolate, *Saccharomyces cerevisiae* KNU5377

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Abstract The difference in the thermotolerance between *Saccharomyces cerevisiae* KNU5377 and ATCC24858 was compared by assaying the amounts of trehalose accumulated under growth and heat shock conditions. Both strains exhibited similar trehalose accumulation during the growth period, but an intrinsic thermotolerance was much higher in KNU5377 than in the control strain. This result implied that some strain-specific characteristics of KNU5377, other than trehalose accumulation, primarily were responsible for its higher intrinsic thermotolerance. Heat shock at 43°C for 90 min to the exponentially growing cells resulted in the maximum level of trehalose in both strains. Trehalose accumulated at least twice more in KNU5377 by the heat shock than in the control, due to the maintenance of its neutral trehalase activity even after the heat shock. Consequently, the increase of acquired thermotolerance in both strains correlated with an increase in the trehalose content in each strain. In conclusion, KNU5377 exhibited a well-modulated trehalose-related mechanism to accumulate more trehalose by means of maintaining neutral trehalase activity after heat shock than the control strain, thereby contributing to its acquired thermotolerance.

Key words: *Saccharomyces cerevisiae*, trehalose, neutral trehalase, thermotolerance

Saccharomyces cerevisiae KNU5377, originally designated as F38-1 [6, 7], was isolated as a thermotolerant yeast that can produce fuel alcohol under stress conditions, especially at a high temperature like 40°C. Due to its tolerance, this strain is expected to have potential applications in the alcohol fermentation industry. Therefore, the elucidation of the mechanisms causing its thermotolerance may provide strategies for further improving its tolerance and industrial applications.

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S. cerevisiae cells exposed to mild thermal stress synthesize stress proteins and undergo physiological changes including trehalose accumulation, and as a result, acquire thermotolerance at lethal temperatures. This phenomenon is known as acquired thermotolerance. However, if cells were tolerant to thermal stress without the induction of heat shock responses, the phenomenon is called intrinsic thermotolerance. For example, cells at the stationary phase undergo physiological and morphological changes, leading the cells into an intrinsically more thermotolerant state than in the exponentially growing phase [15]. Many investigators have abundantly described the concept of acquired thermotolerance, but there is little information for that of intrinsic thermotolerance [2, 3].

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a type of disaccharide synthesized as a stress protectant in many eukaryotes [1, 15, 16]. Originally, trehalose was thought to serve as a storage carbohydrate [18], however, this perception has been changed to one of stress protectant, based on an analysis of glycogen and trehalose accumulation in *S. cerevisiae* [15].

It has also been reported that trehalose is accumulated in cells under environmental stress and appeared to stabilize macromolecules, including proteins and plasma membrane, implying that it plays an important role in yeast thermotolerance [4, 5, 9, 12, 13, 17]. However, it has also been suggested that trehalose hydrolysis rather than trehalose accumulation is more important for acquiring thermotolerance [11]. The importance of trehalose in stress tolerance *in vivo* has already been demonstrated, when trehalose levels correlated with cell survival under lethal conditions. In addition, cellular trehalose concentrations also increased in response to elevated temperatures, ethanol, desiccation, freeze-thaw, and hydrogen peroxide [14, 16].

Trehalose is known to be degraded by various trehalases, including neutral trehalase in cytosolic compartments and acid trehalase in vacuoles, which hydrolyze the disaccharide to yield two molecules of glucose. During recovery from heat shock or exit from the stationary phase, the primary

responsibility for trehalose hydrolysis rests with the cytosolic trehalase; neutral trehalase 1 (Nth1p) [11]. The expression of the neutral trehalase genes, including *NTH1* and *NTH2*, occurs constitutively and increases at elevated temperatures, leading to the paradoxical situation that the enzymes responsible for both trehalose synthesis and trehalose hydrolysis are further induced by heat stress [17].

Accordingly, the current study was undertaken to investigate the trehalose metabolism to elucidate the mechanism responsible in both the intrinsic and acquired thermotolerance of constitutively thermotolerant and relatively thermosensitive *S. cerevisiae* strains in relation to their thermotolerance levels.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

Saccharomyces cerevisiae KNU5377 (abbreviated as KNU5377 hereafter) was isolated from sewage soil [6, 7], while *S. cerevisiae* ATCC24858 was used as a wild-type control, as it has been reported to be ethanol tolerant among the strains of *S. cerevisiae* in the American Type Culture Collection and found by the current authors to be a moderately thermotolerant strain (unpublished data). Both strains were grown with aeration in YPD (yeast extract 1%, peptone 2%, glucose 2%) media at 30°C.

Heat Shock and Recovery Conditions

To analyze the changes of the trehalose content and neutral trehalase activities, the cells were first heat shocked for 90 min, transferred immediately to 30°C, then incubated further for 60 min. During the heat shock and recovery period, a total of 150 min, cells were withdrawn at 30 min intervals, and the trehalose content and neutral trehalase activity were analyzed. The heat shock temperatures used for analysis of the trehalose accumulation and neutral trehalase activity were 37, 40, 43, and 48°C, with 30°C as the control.

Viability Assay

The cell viability was determined as the % of survivors calculated from the colony forming units (CFUs) before and after exposing the cells to 48°C for 60 min in a water bath, and the data are illustrated as logarithmic values in graphs. One ml of cells under various conditions, including the incubation time and heat shock/recovery period, were withdrawn using a microcentrifuge tube, then 0.5 ml of the cell sample was immediately transferred to 48°C for 60 min, while the remaining 0.5 ml in the microcentrifuge was stored on ice. After the heat treatment, the heat-treated and nontreated cells were serially diluted to determine the viability, and spread on YPD agar media to make 100–200 colonies per plate. After culturing overnight, surviving cells were counted and the % of survivors were calculated.

Trehalose Content Analysis

To investigate trehalose content during the growth period of 24 h, the cells were withdrawn at intervals of 2 h. For the heat shock and recovery conditions, exponentially growing cells were transferred to fresh YPD media, and then cell samples were prepared according to the procedures as described above for heat shocked and recovery conditions. The cell suspensions (1 ml) were boiled for 60 min, and the cytosol was prepared to treat with trehalase (α, α-trehalose glucohydrolase, EC 3.2.1.28., Sigma Co., St. Louis, U.S.A.), as reported [10], to hydrolyze the intracellular trehalose stoichiometrically. Subsequently, the liberated glucose was determined enzymatically using a glucose assay kit (Boeringer Mannheim Co. #716251, Mannheim, Germany). The trehalose content (μg trehalose synthesized by heat shock/mg of DCW) was defined as the [(μg trehalose of sample)-(μg trehalose of control)]/mg of dried cell weight. The control represented in this equation denotes a cell that was not treated by the trehalase.

Neutral Trehalase Activity

The cells prepared as described in the preceding section were disrupted with glass bead (0.5 mm diameter) at 4°C in imidazole buffer (50 mM, pH 7.0 containing 10 mM PMSF), then the crude neutral trehalase-containing fraction was extracted by removing the cell debris with centrifugation (10,000 rpm for 2 min). The total protein concentration in the fraction was determined using a protein assay kit (Bio-Rad Inc., DC # 500-0116, CA, U.S.A.). The reaction mixtures consisted of 0.1 M trehalose and crude trehalase in the imidazole buffer. After allowing the enzymatic reaction at 37°C for 20 min, the reaction was terminated by boiling the mixture for 5 min, and the liberated glucose concentration was measured using the glucose assay kit, as mentioned above. The specific activity unit (U) was defined as the degraded trehalose concentration (μg ml^{-1}) per total protein concentration (μg ml^{-1}) over 20 min. One unit (neutral trehalase)=[μg/ml trehalose (sample-control)]/ total protein (μg/ml). Here, the control denotes the glucose concentration in a cell without the above enzymatic reaction.

RESULTS AND DISCUSSION

Trehalose Content and Intrinsic Thermotolerance

The mobilization of the trehalose accumulation, neutral trehalase activity, and viability were compared and analyzed relative to the difference in the thermotolerance between *S. cerevisiae* KNU5377 and ATCC24858. In particular, the effect of trehalose on an intrinsic thermotolerance of *S. cerevisiae* KNU5377 was investigated during a growth period of 24 h.

The trehalose contents in both strains were negligible during the exponential growth phase, corresponding to the first 10 to 12 h, then started to accumulate from the late

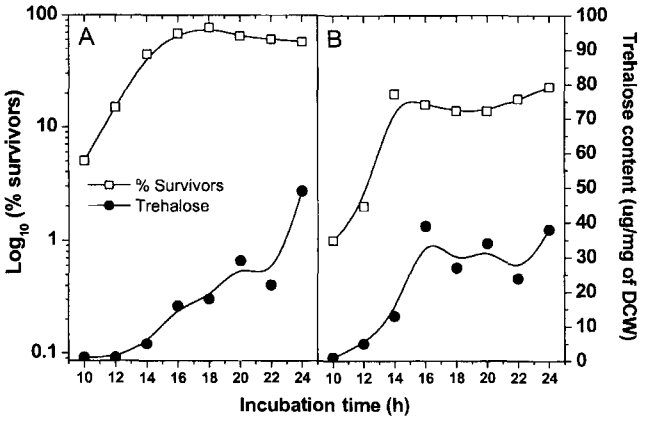

Fig. 1. Trehalose content and thermotolerance of *S. cerevisiae* KNU5377 and ATCC24858 under growth conditions.
The cells were aerobically cultured in YPD media for 24 h at 30 °C. The viabilities and trehalose contents were determined every 2 h. After lethal heat stress at 48°C for 60 min, the viability was determined by using colony forming units (CFUs) and expressed as $\log_{10}$(% of survivors). The open squares and closed circles denote the viabilities and trehalose contents, respectively. Panels A and B exhibit data for *S. cerevisiae* KNU5377 and ATCC24858, respectively.

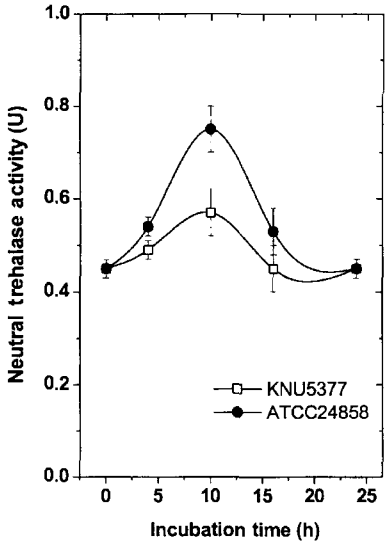

Fig. 2. Change in neutral trehalase activity under growth conditions.
The experimental conditions were the same as in Fig. 1. The squares and circles denote *S. cerevisiae* KNU5377 and ATCC24858, respectively.

logarithmic growth phase after about 12 h (Fig. 1). The viability in both strains was also increased during the growth, exhibiting a similar pattern with the change of trehalose content. Therefore, the increasing pattern in viability exhibited a parallel relationship with the trehalose accumulation in both strains. This overall pattern of the changes in thermotolerance level and trehalose accumulation was also consistent with the results obtained on the trehalose accumulation in *S. cerevisiae* strains [15, 17].

The trehalose-degrading enzyme, neutral trehalase, showed a pattern opposite to the trehalose accumulation through the growth period. That is, the neutral trehalase activity was elevated in the exponential growth phase and slightly lowered in the stationary phase (Fig. 2), thereby providing the mechanism for the change in the trehalose content during the growth period in both strains. However, there was a difference in the neutral trehalase activities between these two strains, in which the control strain exhibited higher activity than the KNU5377, especially at the exponential growth phase. This result suggested that the relatively low activity in KNU5377 was enough to degrade the trehalose in exponential growth phase.

Consequently, KNU5377 exhibited a higher viability than the control strain all through the growth period of 24 h (Fig. 1), implying that KNU5377 was constitutively thermotolerant. Although it was considered for the effect of stationary phase on the trehalose and thermotolerance, a high viability in KNU5377 during the whole growth period indicated that there should be some factors which were required for introducing its intrinsic thermotolerance, other than trehalose accumulation and the effect of the stationary phase. It means that, although the changes of thermotolerance in both strains were in part correlated to trehalose metabolism, the intrinsic thermotolerance in KNU5377 was not dependent only on the difference of trehalose contents in the strains *in vivo*, but possibly affected by unknown mechanisms required for introducing its intrinsic thermotolerance.

It was concluded in this study that the trehalose accumulation during the growth was parallel with the increase of thermotolerance in both strains, but it could not explain the higher intrinsic thermotolerance in KNU5377, because there was a similar trehalose accumulation during the growth period. Instead, other factors rather than trehalose accumulation might be primarily related to the intrinsic thermotolerance of KNU5377, and the higher intrinsic thermotolerance in KNU5377 did not seem to be originated from a constitutively higher accumulation of trehalose than the control strain.

Trehalose Content and Acquired Thermotolerance

Trehalose can be accumulated by entering into the stationary phase as well as by heat shock [1, 4]. Mild heat shock is known to cause stress response in cells, and as a result the cells inductively acquire thermotolerance [10]. Trehalose accumulation is also included in cellular stress responses as a stress protectant, along with a heat shock protein [14, 15].

To induce trehalose accumulation, exponentially growing cells were heat shocked at different temperatures, including 30, 37, 40, 43, and 48°C, then immediately transferred to 30°C for 60 min to allow the increased trehalose contents to be reduced to its original content.

During the heat shock and recovery period, the trehalose content in both strains was maximal at 43°C for 90 min. In particular, at least twice the amount of trehalose was

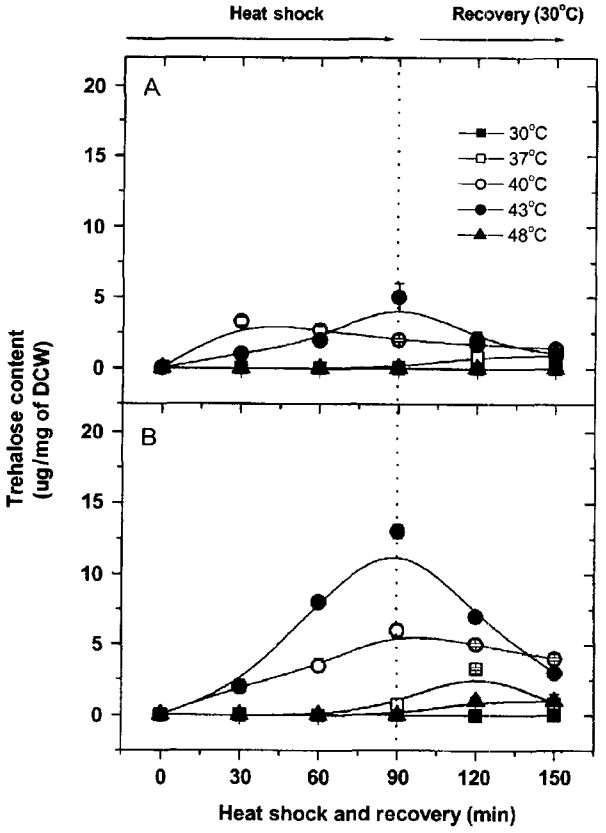

Fig. 3. Trehalose accumulations during the heat shock and recovery period.
Heat shock was applied for 90 min at 37°C, 40°C, 43°C, and 48°C, and at 30°C as a control, then the cells were immediately transferred to 30 °C and further incubated for 60 min. Samples were collected every 30 min. The closed squares, open squares, open circles, closed circles, and closed triangles denote the heat shock temperatures at 30, 37, 40, 43, and 48 °C, respectively. Panels A and B correspond to *S. cerevisiae* ATCC24858 and KNU5377, respectively.

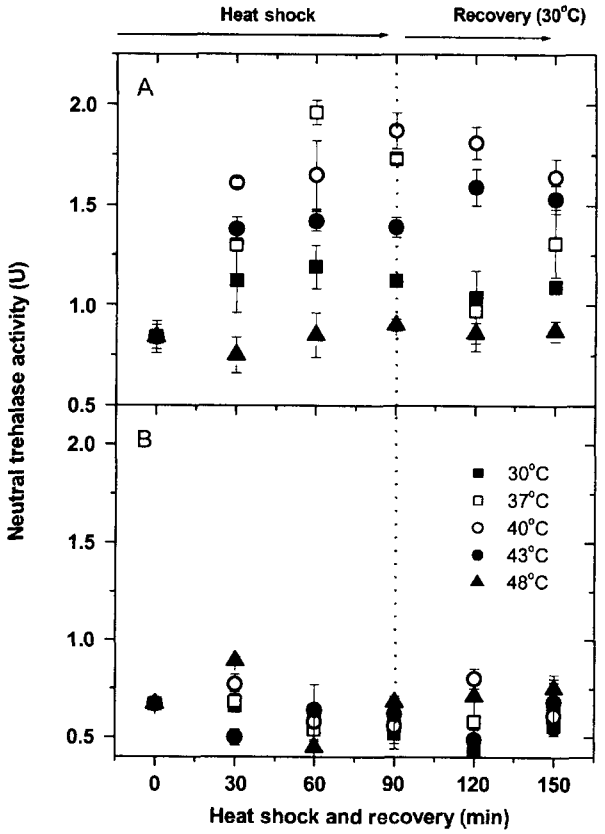

Fig. 4. Neutral trehalase activity during the heat shock and recovery period.
The experimental conditions were identical to those in Fig. 3, and the symbols are the same. Panels A and B exhibit the data for *S. cerevisiae* ATCC24858 and KNU5377, respectively.

accumulated at 43°C for 90 min in KNU5377 than in the control (Fig. 3). Then, the trehalose accumulated by the heat shock was actually decreased by transferring the cells to 30°C. This change in the trehalose content during the heat shock and recovery period was consistent with previous reports that the disaccharide was inductively accumulated by the heat shock, and then subsequently degraded when the cells were transferred to the optimum growth temperature [11, 17].

Heat shock to the control strain caused a significant increase in the neutral trehalase activity, however, not in the KNU5377 strain. Despite heat shock, KNU5377 rather maintained its original activity. As such, this constant activity in KNU5377 would seem to explain the accelerated accumulation of trehalose after heat shock (Fig. 3B). Meanwhile, the neutral trehalase activity of the control strain, *S. cerevisiae* ATCC24858, was maximized at 37°C, which is known to be the optimum condition for its activity [17].

Using the heat shock at 43°C to induce the maximum amount of trehalose, the viability and trehalose accumulation in the exponentially growing cells were examined and compared with each other (Fig. 5). The exposure of heat shock at 43°C to exponentially growing cells elevated the viability of KNU5377 to a maximum survival of 80% that was increased almost 10 times higher than the viability before the heat shock, resulting in an increment of about 70%. The viability of ATCC24858 after the heat shock was also elevated, yet with a maximal survival of no more than 40%, that was increased about 10 times higher than that before the heat shock, resulting in an increment of about 35%. Accordingly, these results implied that the acquired thermotolerance of both strains induced by heat shock was well correlated with the amount of trehalose accumulated by the heat shock.

An intriguing feature on the neutral trehalase activity in KNU5377 was no induction of the increased activity by the heat shock. Heat shock is generally known to increase the synthesis of neutral trehalase, and as a result, cells acquire an elevated enzymatic activity [11]. However, KNU5377 did not exhibit any increase of its activity at all temperatures examined in this study, suggesting that KNU5377 probably has a defect or different regulatory

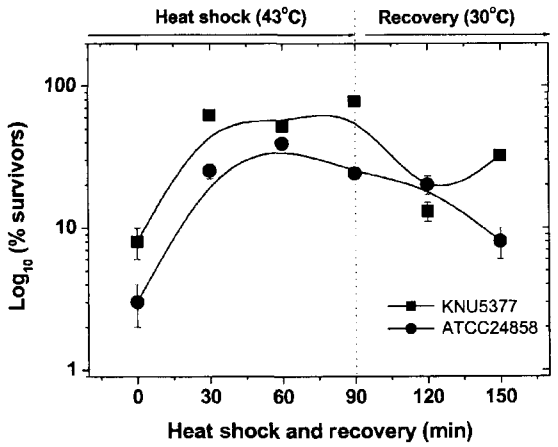

Fig. 5. Viability assay during the heat shock and recovery period. During the heat shock at 43°C for 90 min and recovery period at 30°C for 60 min, the cells were withdrawn at 30-min intervals, then treated with lethal heat stress at 48°C for 60 min to determine the viability. The squares and circles denote *S. cerevisiae* KNU5377 and ATCC24858, respectively.

mechanism on the metabolisms related to this enzyme. Whatever it might have been, the extraordinary event in KNU5377 led the cells to accumulate at least 2 times higher amount of trehalose than in the control strain. Therefore, no induction of neutral trehalase activity only in KNU5377 must be one of major features in KNU5377 to acquire the higher thermotolerance conferred by heat shock at 43°C.

This study on thermotolerant *S. cerevisiae* KNU5377 suggested that there were unknown routes to obtain a higher intrinsic thermotolerance in KNU5377 than the relatively thermosensitive *S. cerevisiae* ATCC24858, since trehalose was not closely related to the intrinsic thermotolerance in KNU5377. For the acquired thermotolerance, KNU5377 exhibited a well-modulated trehalose metabolism to accumulate more trehalose than the control strain by maintaining neutral trehalase activity even after heat shock, thereby contributing to its acquired thermotolerance.

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