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NOTE

Trehalose accumulation enhances tolerance of *Saccharomyces cerevisiae* to acetic acid

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Trehalose confers protection against various environmental stresses on yeast cells. In this study, trehalase gene deletion mutants that accumulate trehalose at high levels showed significant stress tolerance to acetic acid. The enhancement of trehalose accumulation can thus be considered a target in the breeding of acetic acid-tolerant yeast strains.

[**Key words:** Stress tolerance; Trehalose accumulation; Acetic acids; Propionic acid; *Saccharomyces cerevisiae*; Bioethanol production]

Tolerance to organic acids in yeast has significant advantages for bioprocesses such as bioethanol production. It is known that acetic acid is generated from acetylated hemicellulose and strongly inhibits the fermentation ability of *Saccharomyces cerevisiae* during ethanol production from lignocellulose, which is considered a suitable feedstock in second-generation bioethanol production (1). We consider that the utilization of *S. cerevisiae* with high acetic acid tolerance can prevent stuck fermentation even in the presence of acetic acid. In addition, we have proposed a bioprocess combining organic acids as a bacteriostatic agent and a yeast strain that is tolerant to organic acids (2,3). One of the advantages of this process is that it eliminates the need for antibiotics to prevent bacterial contamination. This is crucial, since antibiotics such as virginiamycin are still being used in commercial bioethanol production today (4), despite their undesirability from an ecological viewpoint. Unlike many other wastes generated during bioethanol production, antibiotics cannot be recycled as useful byproducts like forage or fertilizer (3). Propionic acid, in contrast, is usually used as a food preservative (5,6).

In *S. cerevisiae*, the uptake of organic acids occurs both by facilitated diffusion *via* the Fps1 channel (7) and by passive diffusion. At natural cytosolic pH, however, the dissociation of the acids leads to the release of protons and the respective anions, which induce the intracellular acidification and inhibition of fermentation ability (8). Haa1, a transcriptional activator, is considered to be responsible for acetic acid responses (5,6). Approximately 80% of the genes induced by acetic acid addition are regulated by Haa1 (6). Haa1 binds to an acetic acid-responsive element (ACRE), activating the expression of several targets, including membrane transporter genes *TPO2* and *TPO3* (6). Recently, we reported that *S. cerevisiae* overexpressing *HAA1* acquired a higher level of acetic acid tolerance (9).

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside), which is a non-reducing disaccharide of glucose, is considered one of the most important molecules protecting against environmental stresses in *S. cerevisiae*. The most significant function of trehalose is to protect proteins and lipids included in the membrane structure against different kinds of stress conditions, such as heat and freeze–thaw (10). Cellular levels of trehalose are controlled by an enzymatic balance between its synthesis enzymes (Tps1 and Tps2) and degradation enzymes (Nth1 and Nth2) (11,12). Nth1 activity is regulated by the Ras/adenylate cyclase signal-transduction pathway, which converts the inactive form into the phosphorylated active form (10,13). It has been reported that the deletion mutants of *NTH1* accumulate higher levels of trehalose relative to their respective parent strains and tolerate environmental stresses such as high temperature, freeze–thaw, and high osmolality (10,13,14). As far as we know, detailed analyses of the correlation between trehalose and organic acid tolerance have not been performed. In this study, we show that there is a strong correlation between the trehalose accumulation level and organic acid tolerance, and we demonstrate that trehalose accumulation fully compensates for acetic acid sensitivity due to the deletion of *HAA1*.

The *S. cerevisiae* strains used in this study are listed in Table S1. Because differences in auxotrophy could affect the estimation of organic acid tolerance in *S. cerevisiae*, we used yeast strains that have the same auxotrophy. Yeast cells were cultivated in liquid YPD medium [2% glucose, 1% yeast extract (Difco Laboratories, Detroit, MI, USA) and 2% peptone (Difco)] or YPD agar medium containing 2% agar at 30°C, unless otherwise stated. For the fermentation evaluation (Fig. 2), YP5D medium (5% glucose, 1% yeast extract and

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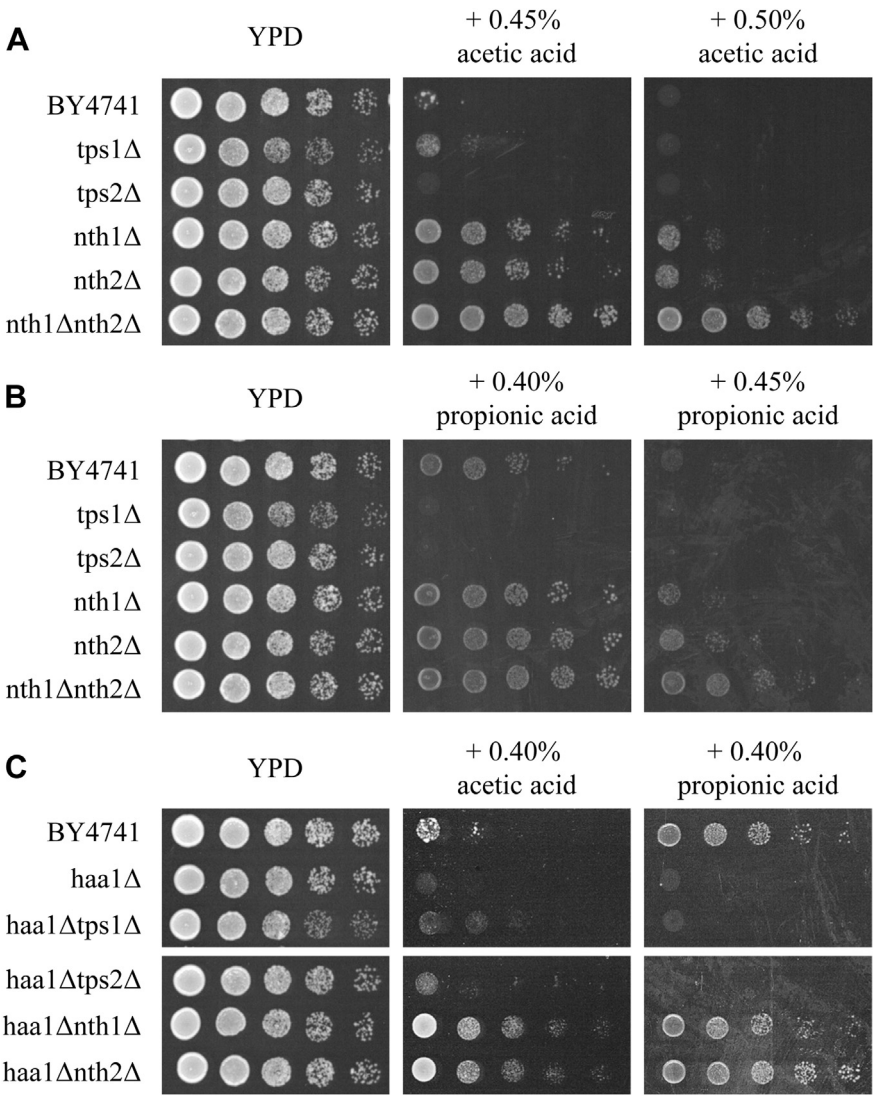

FIG. 1. Organic acid stress tolerance of the mutants of trehalose metabolisms from a wild-type background (A, B) and the mutants of trehalose metabolisms from a *haa1* background (C). Panel A shows the results of an assay using 0.45% and 0.5% acetic acid, and panel B, 0.40% and 0.45% propionic acid, to determine the level of tolerance to organic acids between trehalose accumulation mutants and the wild-type strain (BY4741). Panel C shows the results of an assay using 0.40% acetic acid and 0.40% propionic acid to compare the level of tolerance to organic acids between haa1Δ and haa1Δ with trehalose accumulation mutations, and the wild-type strain (BY4741). Approximately 10^5 cells and serial dilutions of 10^{-1} to 10^{-4} (from left to right) of each strain were spotted on the plates shown in each panel and incubated at 30 °C.

2% peptone) was used. In the trehalose-loading experiment (Fig. S1B), SD medium (2% glucose, 0.17% yeast nitrogen base w/o amino acids, 0.5% ammonium sulfate, 0.5% casamino acids and 0.13% amino acid dropout mixture) was exclusively used because we wanted to avoid media containing yeast extract, which generally includes trehalose. In this experiment, casamino acids and amino acid dropout mixture were used in order to enhance the growth rate.

To determine the correlation between the trehalose accumulation level and organic acid tolerance, we employed strains nth1Δ, nth2Δ, tps1Δ, and tps2Δ (Table S1). The trehalose levels of such strains in the exponential phase (i.e., optical density at 600 nm [OD_{600}] = 1.0) were estimated by a method described previously (14). Both nth1Δ and nth2Δ accumulated higher levels of trehalose than tps1Δ, tps2Δ, and wild-type (Fig. S1A). The spot assays for the estimation of acetic acid and propionic acid in YPD medium containing 0.45% and 0.50% acetic acid (pH 4.3; Fig. 1A) or YPD medium containing 0.40% and 0.45% propionic acid (pH 4.5; Fig. 1B) were performed as described previously (5,9). As shown in Fig. 1A and B, nth1Δ and nth2Δ were more tolerant to both acetic and propionic acids than the wild-type strain. In contrast, tps1Δ and tps2Δ were hypersensitive to such organic acids, the levels of which were determined in a medium containing lower levels of acetic (0.45%) or propionic (0.40%) acid (Fig. 1A and B). Based on these results we constructed an nth1Δ nth2Δ double-knockout mutant using the *hphMX6* gene as a selective marker and evaluated its acetic and propionic acid stress tolerance (Fig. 1A and B). The nth1Δ nth2Δ strain showed much higher acetic and propionic acid stress tolerance depending on trehalose accumulation levels (Fig. 1A, B and Fig. S1A), compared with either single-knockout mutant. To the best of our knowledge, these are the first data that show a significant correlation between organic acid stress tolerances and trehalose accumulation. Our results strongly suggest that intercellular trehalose contributed to tolerance to organic acids stresses in *S. cerevisiae*. We focused on acetic acid in the following analyses because the cellular response systems of yeast cells to acetic acid have been investigated in detail (5–7).

To gain insights into the correlation between the Haa1 acetic acid response system and trehalose accumulation, double deletion mutants (haa1Δ tps2Δ and haa1Δ nth1Δ) were constructed by the