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Isolation and Characterization of Salt Tolerant Mutations in Budding Yeast *Saccharomyces cerevisiae*

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Abstract In order to study the mechanism for the adaptation to salt stress, we mutagenized budding yeast *Saccharomyces cerevisiae* with Ethylmethane sulfonate, and isolated salt-tolerant mutants. Among the salt-tolerant mutants, two strains exhibit additional temperature sensitive phenotype. Here, we report that these two salt-tolerant mutants are specific to Na^+ rather than general osmotic stress. These mutant strains may contain mutations in the genes involved in Na^+ homeostasis.

Key words: *Budding yeast, EMS mutagenesis, Salt-tolerance, Na^+ homeostasis*

Introduction

The genetic improvement of salt-tolerance is an urgent need for the agriculture in arid regions. Classical genetic methodologies based on crosses between crop plants and salt-tolerant relatives have already made some progress [8,17], but knowledge of the molecular basis of salt- tolerance will facilitate further progress.

Metabolic systems likely to affect salt-tolerance include osmolyte synthesis [18], ion transport [2,13] at the vacuolar and plasma membranes, and any system sensitive to high intracellular concentrations of salt. Cellular targets of salt toxicity include protein synthesis and some reactions of sugar metabolism, which are salt-sensitive *in vitro* [11]. However it remains still largely unknown what aspect of cellular metabolism is most sensitive to high intracellular salt concentration.

As an approach to the isolation of salt-tolerance genes and to the identification of cellular processes most crucial to salt-tolerance we mutagenized budding yeast *Saccharomyces cerevisiae*, and isolated salt-tolerant mutants. Here, we report isolation and characterization of the salt-tolerant mutations.

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Materials and Methods

strains, media, and genetic methods

The yeast strain used in this study is DBY1826 (*MATa ade2 his3 leu2 ura3*). Yeast genetic manipulations as well as the preparation of rich medium YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose) were performed as described by Rose *et al* [16]. Cells were routinely grown at 26℃ unless otherwise specified. YPD solid plates which were used for selecting salt-tolerant mutants contained 1M NaCl.

Yeast mutagenesis and selection for salt-tolerant mutants

DBY1826 yeast strain was mutagenized with Ethylmethane Sulfonate (EMS) as described by Lawrence [14]. Overnight culture of yeast cells (2.5 ml, 2×10^8 cells/ ml) was washed twice with 50 mM potassium phosphate buffer, PH 7.0, and resuspended in 10 ml of the same buffer. For EMS mutagenesis, 0.3 ml of EMS was added to the 10 ml of the cells, and the cells were incubated at 26℃ for the indicated time in order to obtain 50 to 60% killing rate. The mutagenesis reaction was stopped by adding 0.3 ml of a freshly made 10% (w/v) filter- sterilized solution of sodium thiosulfate, and wased them twice with sterile water. The killing rate was determined by plating EMS treated cells with appropriate dilution on YPD plate, and counting the numbers of survived colonies. The salt-tolerant mutant cells were selected by seeding YPD plate containing 1M NaCl with about 2×10^7 EMS treated cells per plate, and incubating for 3-5 days at 26℃.

Salt-tolerance test for the isolated mutants

Tolerance to NaCl, LiCl, or KCl was tested as described in Glaser *et al* [10] with minor modifications. Ten $\mu\ell$ of saturated cultures was inoculated in 5 ml YPD liquid medium supplemented with 0.15M LiCl, 0.7M NaCl, 0.7M KCl or 1.0M sorbitol. Growth was recorded by turbidity

measurements after 15h.

Results and Discussion

EMS mutagenesis and survival curve

Mutation study was widely used to investigate the function of a gene. In most cases of mutagenesis, appropriate mutagen dose and mutagen-treating time are a important factor to obtain a proper mutation. Too high mutagen dose would kill most of the mutagen treated cells, and the survived cells may contain multiple mutations. However, when too low dose of mutagen was used, enormous cells should be screened to get desired mutants.

We mutagenized yeast strain DBY1826 as described in Materials and Methods section to obtain salt-tolerant mutants. The killing rate of the mutagen was determined by plating the mutagen-treated cells onto YPD plate, and counting the survived colonies. As shown in Fig. 1, when the cells were incubated with EMS for 20 min, survival rate was reduced to 50% compared to that of untreated cells. When the EMS treating time was increased to 30 min, approximately 70% of the cells was killed. We, therefore, incubated the cell with EMS for 20 and 25 min to get 40 to 50% survival rate, respectively.

Isolation of salt-tolerant mutants

The EMS treated yeast cells were plated on YPD containing 1M NaCl, and incubated at 26℃ for 3-5 days. Initially salt-tolerant colonies obtained were streaked on YPD plate, and retested for salt-tolerance by transferring the cells onto YPD plate containing 1M NaCl. As shown in Fig. 2, ten salt-tolerant strains were isolated.

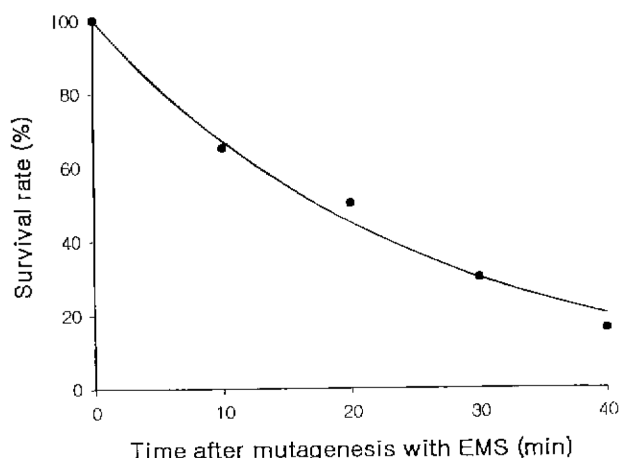

Fig. 1. Effect of EMS on growth of yeast cells (DBY1826) were treated with EMS for indicated time intervals. Survival rate was determined by plating EMS treated cells with appropriate dilutions on YPD plates, and counting the numbers of survived colonies.

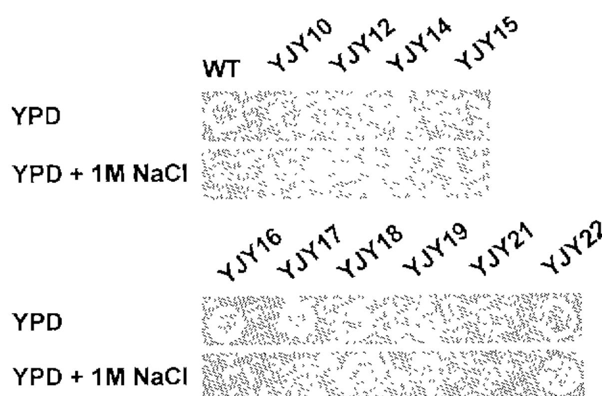

Fig. 2. Growth phenotype of salt-tolerant mutants. Suspensions of the indicated yeast strains were spotted on YPD plate or YPD plate containing 1.0M NaCl, and allowed to grow at 26℃ for 2 days.

A prominent feature of the cellular response of yeast cells to an increase in the external salt concentration is the enhanced production and accumulation of the polyhydroxyalcohol glycerol [3,4,5]. A key enzyme in this process, in *Saccharomyces cerevisiae*, is glycerol-3-phosphate dehydrogenase, which is encoded by *GPD1* gene, and cells have been shown to respond to the salt stress by increasing specificity of this enzyme [4,7].

The components of a signal transduction pathway leading to the transcriptional activation of the *GPD1* by changes in the extracellular osmolarity have been identified [6]. This high-osmolarity glycerol response (HOG) pathway contains a mitogen-activated protein kinase (Hog1) and the mitogen-activated protein kinase kinase (Pbs2). Tyrosine phosphorylation of Hog1 in response to osmotic stress is prerequisite for the transcriptional activation of the GPD1 [1].

In additon to intracellular glycerol accumulation, maintaining Na^+ homeostasis achieved by the coordinate regulation of plasma membrane-located influx and efflux system is also important in order to respond to the NaCl stress. Under NaCl stress, the K^+ uptake system is converted to a high affinity mode that results in higher K^+-Na^+ discrimination, which reduces the Na^+ ion [12]. Na^+ efflux is mediated by the P-type ATPase encoded by *ENA1* [9]. These NaCl-induced cellular responses involved in ion homeostasis are under control of a signalling pathway which contains the Ca^{2+}-calmodulin-dependent protein phosphatase, Calcineurin [15].

The salt-tolerant mutants we isolated possibly contained mutations which could lead to the activation of the genes that are involved in the adaptation process to the high extracellular osmolarity.

Secondary phenotype of YJY14, YJY15 strains

In order to further study the salt-tolerant mutations, we tried to test the mutants whether they exhibit additional

recessive phenotypes which can be used to identify the nature of the mutations. Indeed, among the ten salt-tolerant mutants, two strains (YJY14 and YJY15) showed Ts^- growth phenotype when incubated at 37℃ (Fig. 3). Other mutants did not show any growth defect at either 37℃ or 13℃ (data not shown). Therefore, we concentrate these two salt-tolerant mutants carrying additional Ts^- phenotype for further studies.

NaCl, LiCl, KCl or sorbitol tolerance

To test whether these two mutants are specifically tolerant to Na^+ ion or generally tolerant to other ions and osmolytes, their growth rate in YPD medium containing increasing amounts of KCl, LiCl, NaCl, or sorbitol were compared. The result, shown in Fig. 4, shows that both of the strains were tolerant to NaCl and LiCl (Fig. 4A and 4C), but did not show any significant tolerance to either KCl or sorbitol (Fig. 4C and Fig. 4D). YJY15 strain appeared to be rather sensitive to KCl. These results suggest that these salt-tolerant strains we isolated are specifically tolerant to Na^+ ion since LiCl is generally used to substitute NaCl for salt toxicity test. Further characterization of these salt-tolerant mutations will elucidate the mechanism in which yeast cells adapt to environmental salt stress.

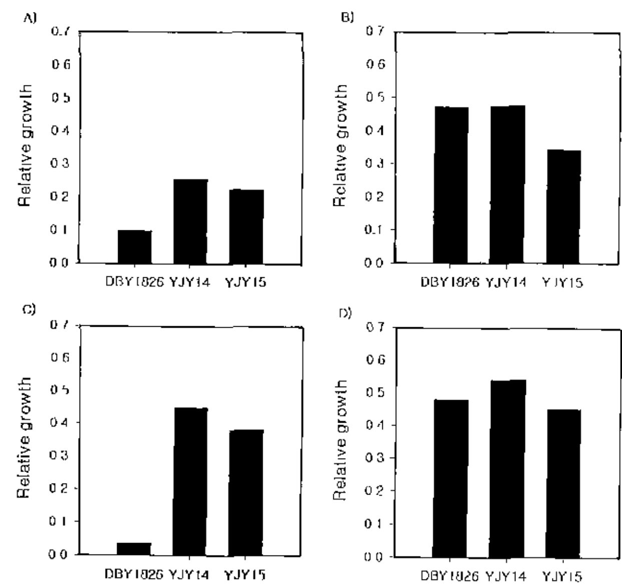

Fig. 4. Effect of different solutes on growth of salt- tolerant mutants. Yeast cells were grown in the YPD medium containing indicated amount of various solutes, and growth was recorded by turbidity measurements after 15hr. Relative growth of each strains was determined by comparing growth in YPD with growth in YPD containing indicated amount of different solutes. A; 0.7M NaCl, B; 0.7M KCl, C; 0.15M LiCl, D; 1.0M Sorbitol.

Acknowledgment

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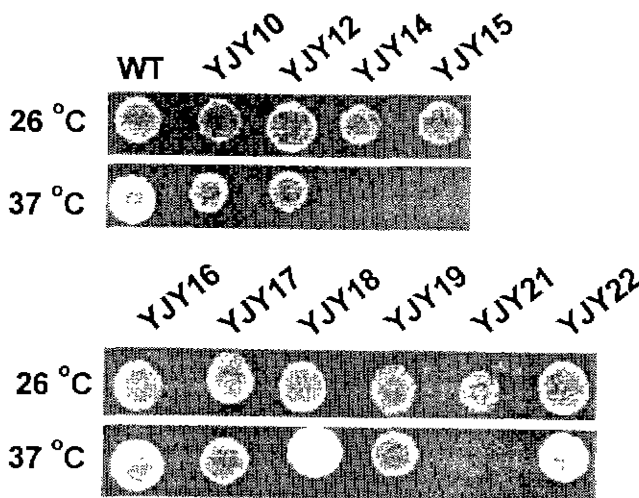

Fig. 3. Ts^- growth phenotype of salt-tolerant mutants. Suspensions of the indicated strains were spotted on YPD plate, and allowed to grow either 26℃ or 37℃ at indicated temperature for 2 days.

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