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Overexpression of *Saccharomyces cerevisiae* mannitol dehydrogenase gene (YEL070w) in glycerol synthesis-deficient *S. Cerevisiae* mutant

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Abstract

Saccharomyces cerevisiae ORF YEL070w encodes mannitol dehydrogenase (MDH), as confirmed by the present results. Six highly homologous peptide motifs were identified in the amino acid sequence of the YEL070w product by comparison with those of microbial MDH proteins. In particular, a typical coenzyme-binding site and an active site that are specific to enzymes participating in the dehydrogenation of alcohols groups were identified in these homologous peptide motifs. *S. cerevisiae* cells overexpressing YEL070w exhibited MDH activity showing high substrate specificity for mannitol and a coenzyme requirement of NAD^+ in the oxidation reaction. The salt-sensitive phenotype of *S. cerevisiae* glycerol-synthesis-deficient mutant (*ΔGPD1* and *ΔGPD2*) was complemented and tolerance to several stressors (i.e., heat and sugar) was observed with YEL070w expression.

Keywords: Yeast; Mannitol dehydrogenase; Salt tolerance; *Saccharomyces cerevisiae*

1. Introduction

Mannitol is a six-carbon sugar alcohol that is present in various species of fruit, vegetables and algae, and is synthesized by a few fungi and bacteria. Mannitol is also widely used in the food and pharmaceutical industries.

Mannitol dehydrogenase (MDH) has been purified from bacteria belonging to the genus *Lactobacillus* [1,2]. MDH has also been purified from *Psudomonas fluorescens* [3], *Aspergillus parasiticus* [4], *Candida magnoliae* [5] and *Absidiaa glauca* [6]. MDH genes have been cloned from many bacteria, including *Agrobacterium tumefaciens* [7], *Brucella melitensis* [8], *Leuconostoc mesenteroides* [9], *L. psudomesenteroides* [10], *P. aeruginosa* [11], *P. fluorescens* [12], *Rhodobacter sphaeroides* [13] and *Sinorhizobium meliloti* [14], as well as the fungal pathogen *Gibberlla zeae* [15], button mushroom (*Agaricus bisporus* [16]) and celery (*Apium graveolens* [17]). Mannitol metabolism in organisms other than yeast was recently reviewed by Iwamoto and Shiraiwa [40].

Studies on the MDH gene have been carried out in yeast species. By searching the yeast MDH gene database of the DDBJ and the yeast genome database, two ORFs were found to correspond to *Saccharomyces cerevisiae* MDH. YEL070w is present on chromosome V [18] and YRN073c is located on chromosome XIV [19]. Their amino acid sequences were found to be 100% homologous. To understand the significance of mannitol in the pathobiology of the pathogenic yeast *Cryptococcus neoformans*, Perfect et al. identified the MDH gene from *C. neoformans* (*CnMDH*), which confers the ability of *S. cerevisiae* to grow on mannitol with substantial NAD^+-dependent MDH activity [20]. Recombinant yeast having the *CnMDH* gene exhibited MDH activity. However, the MDH gene did not encode the cryptococcal MDH gene, but rather directed the cryptic expression of *S. cerevisiae* MDH in this strain. It was also demonstrated that the amino acid sequence of the MDH protein purified from *S. cerevisiae* cells harboring the *CnMDH* gene was identical to that of the hypothetical *S. cerevisiae* ORF YEL070w identified by *S. cerevisiae* genome analysis [18].

Osmotic regulation in yeast cells is accomplished by accumulation of osmolytes such as glycerol and D-arabitol. Glycerol synthesis is regulated by the action of glycerol-3-phosphate dehydrogenase, and a double-gene disruption mutant (YSH642 strain) of these genes (*GPD1* and *GPD2*) is significantly sensi-

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tive to salt [21]. Thus, by introducing a gene related to the synthesis of an osmolyte into YSH642 strain, the resultant recombinant yeast strain would exhibit resistance to salt. In other words, the YSH642 strain is a good recipient for assessing the function of osmolyte-synthesizing genes. Here, in addition to inadequate osmolyte (glycerol) synthesis, the YSH642 strain also lacked MDH activity.

In this study, we found that the expression of *S. cerevisiae* ORF YEL070w in the salt-sensitive YSH642 strain was able to complement the salt sensitivity resulting from deficient glycerol synthesis. We confirmed this observation by detecting MDH activity in the cell-free homogenate of the recombinant yeast, thus demonstrating that YEL070w encodes MDH. Moreover, a yeast strain overexpressing this gene exhibited resistance against a variety of stressors.

2. Materials and methods

2.1. S. cerevisiae and Escherichia coli

S. cerevisiae YSH642 (gift from Prof. S. Hohmann; *Mata gpd1Δ::TRP1 gpd2Δ::URA3 leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2*) and W303-1A (*Mata leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2*) were cultivated in YPAD medium (0.5% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 0.5% (w/v) KH_2PO_4, 0.2% (w/v) $MgSO_47H_2O$, 400 μg/ml adenine (pH 4.8)) and Leu-drop out medium (0.67% (w/v) yeast nitrogen base without amino acid (Difco™; Becton, Dickson and Company, Sperks, MD, USA) containing 2% (w/v) sugar and 1.6 g/l Yeast Synthetic Drop-out Media Supplement with Leucine (Sigma Chemical Co., St. Louis, MO, USA)) with agitation at 30 °C [28]. *E. coli* NovaBlue (EMD Biocsciences Inc., Madison, WI, USA) was cultivated in LB medium (1% (w/v) tryptone, 0.5%(w/v) yeast extract, 1% (w/v) NaCl, pH 7.0) with agitation at 37 °C [37].

2.2. Construction of recombinant plasmids

The DNA fragment containing *S. cerevisiae* ORF YEL070w was prepared by PCR amplification with Ex Taq DNA polymerase (Takara Shuzo Co. Ltd., Otsu, Shiga, Japan) and the following primers: 5′-CAAACTAGTGGAAAAAGAAAAAGTAAC-3′ and 5′-CAAGAGCTCTAGTCATTACTAAG-3′ (underlines indicate *Spe*I and *Sac*I sites). *S. cerevisiae* W303-1A genomic DNA was used as template DNA. After digestion with *Spe*I and *Sac*I, the obtained DNA was inserted into a yeast expression vector (pESC-LEU, Stratagene Co., La Jolla, CA, USA) and introduced into *S. cerevisiae* YSH642 with an Alkali-cation yeast transformation kit (Bio 101, Inc., Vista, CA, USA).

2.3. Assay of recombinant yeast tolerance to stressors

After preculture in Leu-drop out medium containing 2% raffinose at 30 °C, 1/100 volume of preculture was inoculated into Leu-drop out medium containing 1% galactose (in order to express YEL070w under the regulation of the *GAL1/10* promoter), 1% raffinose and various concentrations of NaCl or sucrose. The culture was incubated with agitation at 30 °C. Cell growth was estimated by measuring the optical density at 600 nm [27]. In the heat-tolerance assay, 1/100 volume of preculture was inoculated into Leu-drop out medium. The culture was incubated with agitation at 30 °C until the early stationary phase. The culture was then heated at various temperatures, diluted with sterile water and sprayed onto a YPAD plate with a glass rod. The number of obtained colonies was compared with that derived from unheated control culture.

2.4. Measurement of MDH activity

Recombinant yeast cells harboring ORF YEL070w were cultivated in medium containing galactose (10 ml), and were collected by centrifugation (10,000 × *g*, 5 min). The obtained cell pellet was suspended in 10 mM Tris–HCl buffer (pH 8.0, 1.5 ml). Glass beads (0.5 mm) were added to the cell suspension. The mixture was shaken vigorously with a Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA) twice for 2 min each with occasional cooling on ice. The supernatant was obtained by centrifugation at 10,000 × *g* for 10 min at 4 °C.

For oxidation, the reaction mixture consisted of 0.1 M substrate, 0.1 mM β-NAD^+, and 16.7 mM Tris–HCl buffer (pH 8.5). Absorbance at 340 nm was determined at room temperature [2]. One unit of MDH activity was defined as the amount of enzyme required to reduce one micromole of NAD^+ per minute. For determination of optimal pH for oxidation activity, various 50 mM buffer solutions were used; MES-NaOH (pH 6.0 and 6.5), Tris–HCl (pH 7.0–8.5) and or BIS-Tris–HCl (pH 9.0–11.0). For determination of optimal pH for reduction activity, 50 mM acetate–NaOH (pH 3.5–7.0) was used as a buffer.

2.5. Northern blot analysis of YEL070w transcripts in S. cerevisiae YEL070w-harboring strain

Total RNA was prepared from strains harboring the pESC-LEU plasmid containing YEL070w DNA and the empty pESC-LEU plasmid, as described previously [38]. The DNA fragment described in Section 2.2 was labeled with digoxigenin according to the instructions included with the Digoxigenin DNA Labeling Kit (Boehringer Mannheim Gmbh, Mannheim, Germany) and used as a YEL070w probe. Northern blot analysis was carried out as described previously [39].

3. Results and discussion

3.1. Expression of S. cerevisiae ORF YEL070w

We searched the DNA databases of DDBJ/GenBank/EMBL using yeast mannitol dehydrogenase as a key word. The identified *S. cerevisiae* ORF YEL070w [18] was homologous with the *R. sphaeroides* bacterial mannitol-2-dehydrogenase [13] and the ORF was localized in *S. cerevisiae* genome V [18]. Moreover, *S. cerevisiae* ORF YNR073c was found in the *Saccharomyces* genome database at Stanford University and localized in *S. cerevisiae* genome XIV [19]. The amino acid sequences of the proteins encoded by both ORFs were identical.

The product encoded by *S. cerevisiae* ORF YEL070w consisted of 502 amino acid residues [18]. The number of amino acid residues in MDH varies among bacterial species: *A. bisporus*, 263 residues (belongs to short-chain alcohol dehydrogenase family [16]); *L. pseudomensenteriodes*, 338 residues [10]; *L. mesenteriodes*, 338 residues (belongs to medium-chain alcohol dehydrogenase family [9]); *R. sphaeroides*, 476 residues [13]; *P. aeruginosa,* 491 residues [11]; *P. fluorescens*, 493 residues [12]; *S. meliloti*, 494 residues [14]; *B. melitensis*, 502 residues [8]; and *A. tumefaciens*, 525 residues (belongs to long-chain alcohol dehydrogenase family [7]). The dehydrogenase reductases fall into three main groups that are referred to as the short-chain alcohol dehydrogenases, the medium-chain alcohol dehydrogenases and the long-chain dehydrogenases [5,22]. The yeast enzyme appears to belong to the long-chain alcohol dehydrogenase family.

The amino acid sequences of these MDHs were analyzed by Clustal W (from DDBJ website). The amino acid sequences of five MDHs with high similarity to the protein encoded by YEL070w are shown in Fig. 1. The amino acid sequence of *P.*