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Near-freezing effects on the proteome of industrial yeast strains of *Saccharomyces cerevisiae*

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ABSTRACT

At near-freezing temperatures (0–4 °C), the growth of the yeast *Saccharomyces cerevisiae* stops or is severely limited, and viability decreases. Under these conditions, yeast cells trigger a biochemical response, in which trehalose and glycerol accumulate and protect them against severe cold and freeze injury. However, the mechanisms that allow yeast cells to sustain this response have been not clarified. The effects of severe cold on the proteome of *S. cerevisiae* have been not investigated and its importance in providing cell survival at near-freezing temperatures and upon freezing remains unknown. Here, we have compared the protein profile of two industrial baker's yeast strains at 30 °C and 4 °C. Overall, a total of 16 proteins involved in energy-metabolism, translation and redox homeostasis were identified as showing increased abundance at 4 °C. The predominant presence of glycolytic proteins among those upregulated at 4 °C, likely represents a mechanism to maintain a constant supply of ATP for the synthesis of glycerol and other protective molecules. Accumulation of these molecules is by far the most important component in enhancing viability of baker's yeast strains upon freezing. Overexpression of genes encoding certain proteins associated with translation or redox homeostasis provided specifically protection against extreme cold damage, underlying the importance of these functions in the near-freezing response.

1. Introduction

Low temperature is one of the most important environmental stresses influencing the life and distribution of nearly all living organisms. In the yeast *Saccharomyces cerevisiae*, reductions in ambient temperature have widespread effects on growth and survival, which depend on the severity of the stress. At low but still permissive temperatures (10–18 °C), cold reduces metabolic activity and growth rate. This is relevant for the industrial exploitation of yeast since several fermentations, like brewing and some wine fermentations, take place at around 12–15 °C. When the temperature falls further, near freezing (0–4 °C) or upon freezing, yeast growth stops or is severely limited (Murata et al., 2006). This is exploited for the preservation of microorganisms and yeast-based products, i.e., frozen dough (Randez-Gil et al., 2013), since it is generally assumed that, under these conditions, viability is maintained over long periods of time. Nevertheless, evidence suggests that yeast viability decreases with storage time at 4 °C (Kandror et al., 2004) and especially below 0 °C (Hernández-López et al., 2003; Rodríguez-Vargas et al., 2007). Freezing stress causes serious cell injury, mainly due to the formation of intracellular ice crystals, dehydration and oxidative stress during the thawing process (Aguilera et al., 2007). Despite of this, the cellular responses that protect yeast cells against loss of viability at very low temperature have received little attention.

After a downward shift in temperature, *S. cerevisiae* cells exhibit a genetic and biochemical response. Examination of different microarray-based studies reveals that the transcriptional response to cold is temperature- and time-dependent (Sahara et al., 2002; Homma et al., 2003; Kandror et al., 2004; Schade et al., 2004; Murata et al., 2006). At 4 °C, a significant number of genes associated with the energy and metabolism category, including genes involved in glycolysis (i.e., *HXK1*, *TPI1*, *TDH1-3*, *ENO1/2*, *PYC1/5* and *PDC6*), glycogen (*GAC1*, *GPH1*, *GDB1* and *GLC3*) and trehalose (i.e., *TPS1/2*) biosynthesis are significantly upregulated (Kandror et al., 2004; Murata et al., 2006). *GPD1*, the gene encoding the main enzyme for glycerol production (Albertyn et al., 1994) is also induced at very low temperatures (Panadero et al., 2006). Nevertheless, the

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physiological significance of these changes is unclear. Cold has important consequences in the stabilization of the secondary structure of RNAs that result in the inhibition of gene expression and inefficient degradation of RNAs (Phadtare and Severinov, 2010). A downward shift in temperature also renders ribosomes non-functional in translation. In *Escherichia coli* and other bacteria, a certain group of proteins, the so-called "cold-sock proteins" (Csp), mainly formed by RNA chaperones, are produced to counteract these effects (Thieringer et al., 1998; Gualerzi et al., 2003; Phadtare et al., 2004). However, In *S. cerevisiae*, no proteins homologous to bacterial Csp have been isolated.

Despite of this, evidence suggests that cold-shocked yeast cells induce the translation of specific mRNAs that assist to prevent and alleviate cold-instigated cellular injury. During studies of the adaptation of *S. cerevisiae* to extremely low temperature, Kandror et al. (2004) found that these cells produce large amounts of the disaccharide trehalose due to increased transcription and translation of trehalose-biosynthetic enzymes. Indeed, the relative content of TPS1 and TPS2 enzymes increased markedly in cells exposed to 4 °C and even at 0 °C (Kandror et al., 2004). Moreover, accumulation of trehalose was found to be important for survival at near-freezing temperature or upon freezing. Likewise, exposure of yeast cells to cold activates the biosynthesis of glycerol, which is accumulated in 4 °C-exposed cells providing freeze protection (Panadero et al., 2006). However, the protein changes that favour the accumulation of glycerol are unknown. Whether the increased translation of *TPS1* and *TPS2* mRNAs extends to other cold-induced genes remains unclear. The importance of this response not only in freezing tolerance, but in enhancing viability upon exposure to severe cold stress also needs to be addressed.

Herein, we have performed a proteomic analysis of two industrial baker's yeast strains exposed to near freezing temperatures. Our hypothesis was that extremely low temperatures induce specific changes in the repertoire of yeast proteins and that proteins differentially regulated could be important for yeast cells to survive and adapt to severe cold and freezing conditions. The results uncover new aspects of the cold response which, in addition, may have practical applications in improving resistance of industrial yeast to severe cold stress.

2. Materials and methods

2.1. Strains, culture media and general methods

The baker's yeast strains HS13, L'Hirondelle (LH) and Plus Vital (PV) were used throughout this work. HS13 is a uracil auxotrophic strain derived from a commercial strain (Lesaffre International, Lille, France), while LH and PV are commercial strains produced by the Lesaffre Group and usually employed for general baking. Yeast cells were cultured at 30 °C in defined media, YPD (2% yeast extract [Formedium, Norfolk, UK], 1% peptone [Formedium] and 2% glucose [Formedium]) or SCD (0.2% yeast nitrogen base without amino acids [DIFCO™], 0.5% $(NH_4)_2SO_4$ [Panreac, Barcelona, Spain], 2% glucose). *E. coli* DH5α host strain was grown in Luria–Bertani (LB) medium (1% peptone, 0.5% yeast extract and 0.5% NaCl [Panreac]) supplemented with ampicillin [Formedium] (50 mg/l). Solid media contained 2% agar [Formedium]. Yeast cells were transformed by the lithium acetate method (Ito et al., 1983). *E. coli* was transformed by electroporation following the manufacturer's instructions (Eppendorf, Hamburg, Germany; http://www.masterflex.com/assets/Manual_pdfs/36205-05.pdf). Plate phenotype experiments were made by diluting the cultures to OD_{600} = 0.8 and spotting (3 μl) 10-fold serial dilutions. Unless indicated, colony growth was inspected after 2 and 10–12 days of incubation at 30 and 15 °C, respectively.

2.2. Plasmids

PCR-amplified fragments containing the whole sequence of *EFT1*, *GDH1*, *TAL1*, *TEF1*, *TEF2*, and *TPM1* gene, including its own promoter and terminator were obtained with specific synthetic oligonucleotides [Isogen Life Science, De Meern, The Netherlands] (Supplementary Table S1). The corresponding fragments were first ligated into the pGEM-T Easy Vector (Promega, Madison, WI) or pJET1.2/blunt (Thermo Fisher Scientific, Waltham, MA), then released from this with the appropriate set of enzymes (See Supplementary Table S2) and finally subcloned into the multicopy plasmid YEplac195 (*URA3*; Gietz and Sugino, 1988).

2.3. Viability experiments

Cell cultures were grown in YPD liquid medium at 30 °C (OD_{600} ~ 0.5) and samples were transferred directly to −20 °C, or pre-incubated at 4 °C for 48 h, prior the shift to −20 °C. In both cases, cells were harvested by centrifugation (3000 × *g*, 2 min, 4 °C), washed with distilled water, resuspended in fresh YPD (final OD_{600} ~ 10.0) and 100 μl-aliquots were frozen. At different time points, samples were thawed at 30 °C for 30 min, diluted, and cells were plated onto solid YPD. After 2 days, colonies were counted and the percentage of viable cells was determined.

2.4. Glycerol assay

To determine intracellular glycerol content under cold stress conditions, YPD-grown cells (OD_{600} ~ 0.5) were collected by centrifugation, resuspended in 4 °C-precooled YPD culture medium, and incubated at this temperature. At different times, cells (10 units of OD_{600}) were collected by filtration and quickly transferred to a cold tube containing 1 ml of distilled water. Then, the yeast suspension was boiled for 10 min, cooled on ice, and centrifuged at 15,300 × *g* for 10 min (4 °C). Finally, the supernatant was collected and used for glycerol analysis as described in Panadero et al. (2006).

2.5. Determination of CO_2 in liquid dough

Yeast biomass from HS13 transformants was prepared by cultivating cells (7.6 units of OD_{600}) on molasses plates as previously described (Pérez-Torrado et al., 2010). Final yeast concentration was adjusted to approximately 5 mg (dry weight) per ml. To do this, cell mass was related to optical density measurements at the time of harvest (OD_{600} = 1 equals 0.35 mg cells dry weight/ml). 15 ml of the yeast mixture was poured into a 250-ml screw cap graduated bottle and placed in a 30 °C water bath. Then, 15 ml of 30 °C pre-warmed liquid dough (LD) was added and the suspension incubated at the same temperature. LD solution was prepared as described in Panadero et al. (2005).

Samples for freezing were stored at –20 °C. After 14 days, they were thawed at 30 °C for 30 min before measuring gassing power. In all cases, CO_2 production was recorded for 360 min in a Fermograph II apparatus (ATTO Co., Ltd., Tokyo, Japan).

2.6. Protein extraction and two-dimensional electrophoresis (2DE)

Protein extracts were prepared as described in Blomberg (2002). Soluble proteins were deposited in 18 cm strips of non-linear pH 3–10 and run in the first dimension using a commercial horizontal electrophoresis system (Multiphor II; GE Healthcare, Uppsala, Sweden). Voltage was ramped to 500 V over a period of 5 h, maintained at 500 V for 5 h more, ramped again to 3500 V over a period of 9.5 h and finally maintained at 3500 V for 5 h. The second dimension was carried out in a vertical electrophoresis system (Ettan DALTsix;