

Adenine starvation is signalled through environmental stress response system in budding yeast *Saccharomyces cerevisiae*

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Abstract

In the wild, budding yeast *Saccharomyces cerevisiae* often undergoes periods of nutrient abundance and absolute scarcity. It is capable of effectively halting its cell cycle in G1, in the case of lack of any basic nutrients. However, when lacking metabolic intermediates, the yeast behaves differently. Purine and not pyrimidine auxotrophic starvation in *S. cerevisiae* elicits rapid cell cycle arrest and increase of several stress (oxidative, acid, heat) resistances. Until now, molecular mechanisms governing formation of phenotype during auxotrophic adenine starvation in *S. cerevisiae* are not understood. The aim of the current research was to determine if the elements of environmental stress response system play a role during phenotype formation in adenine starvation in budding yeast. We tested if *MSN2/4* or *RIM15* C-end truncation affects desiccation tolerance in full media and after adenine starvation. We found that functional defects of each element of environmental stress response systems affected desiccation tolerance, however, C-end truncation of *RIM15* lowered desiccation tolerance by several orders of magnitude, while *MSN2/4* C-end truncation only by 2 to 4 times. Therefore, we hypothesize that there are other elements of the environmental stress response system except *MSN4* and *MSN2*, responsible for adenine starvation specific, stress tolerant phenotype formulation.

Key words: adenine starvation, desiccation tolerance, *MSN2*, *MSN4*, *RIM15*, *Saccharomyces cerevisiae*.

Abbreviations: ESR, Environmental Stress Response; PKA, Protein Kinase A; SD, synthetic dextrose; RTG, Retrograde Signalling; TOR, Target of Rapamycin.

Introduction

Starvation is a typical event in the microbial lifetime, and it is estimated that most microorganisms on Earth are in the resting state initiated by lack of carbon/nitrogen/any other source necessary for biomass increase (Gray et al. 2004). In natural settings yeast often undergoes periods of nutrient abundance and absolute scarcity and is capable of effectively reacting to decrease of availability of carbon, sulphur and nitrogen sources. When starving for the aforementioned “basic” nutrients, yeast cells enter a stress resistant phenotype that aids survival till the external nutrient supply is restored (Boer et al. 2010; Petti et al. 2011). For genetic manipulation purposes various auxotrophies (interruptions of amino acid or nucleotide metabolism) have been introduced in yeast. Interestingly, when cells are starved for external supply of those auxotrophic metabolic intermediates (sometimes called “synthetic limitation”), for example, nucleotides or amino acids, yeast cells do not finish their cell cycle and stop division, and they continue to actively metabolize sugars and do not gain stress resistance. This phenotype has been described for uracil and leucine auxotrophic starvation and has earned the description “glucose wasting phenotype” (Boer et al. 2008).

Adenine auxotrophic starvation in many aspects induces a phenotype similar to cells in G0 arrest or quiescence. *Saccharomyces cerevisiae* cells with adenine auxotrophy (*ade8* or *ade2* mutants) when starving for adenine became desiccation tolerant, their budding index declines, and their chronological life span is increased (Kokina et al. 2014). The mechanism how and why cells translate lack of adenine into a desiccation and starvation resistant phenotype is yet unknown.

Various signaling pathways in the yeast cell link nutrient supply to cell growth rate and stress resistance. Target of Rapamycin, (TOR, nitrogen supply), Protein Kinase A (PKA, carbon supply), Retrograde Signalling (RTG) are the prominent examples of such pathways (see review by Broach 2012).

In the case of adenine starvation, we have observed more than a 200-fold increase in desiccation tolerance in *S. cerevisiae* cells (Kokina et al. 2014). Other authors have linked such a huge increase in desiccation tolerance to various genetic factors, including activity of targets of PKA and TOR pathways (Calahan et al. 2011; Welch et al. 2013). *Msn2p*, *Msn4p* and *Rim15p* are components of a system ensuring universal “Environment Stress Response” (ESR). The transcription factors *Msn2p* and *Msn4p* upregulate

genes containing STRE sequences in their promoters (Berry, Gasch 2008). Rim15p is sensor-effector kinase that integrates nutrient and stress signals into widespread ESR response via activating endosulphines Igo1/2p. Those, in turn, provide stability for newly synthesised mRNA (Sarkar et al. 2014). Previously, Msn2/4p together with Rim15p, have been identified as crucial elements ensuring desiccation tolerance in *S. cerevisiae* (Welch et al. 2013).

Our aim was to test if ESR is involved in cell phenotype formation during adenine auxotrophic starvation. To test this, we determined if stable nuclear translocation of Msn2/4p is necessary to ensure adenine starvation mediated desiccation tolerance. We constructed C-end truncations of *MSN2/4* or *RIM15*, checked their identity and effect on growth. We found the cultivation time necessary for complete halt of cell proliferation (adenine starvation), thus giving us a timescale for adenine starvation specific effects. Finally we tested C-end truncation effects on desiccation tolerance of WT and each of newly constructed strains in fast growing and adenine starved cells.

Materials and methods

Yeast strains and genetic constructions

All experiments were carried out using $\Delta ade8$ *S. cerevisiae* strains of CEN.PK2 background (Kokina et al. 2014). Transcription factors were truncated by transforming yeast with linear PCR fragment containing flanking homologous sequences of the respective transcription factor and 6xHis tag and G418 marker in between (Janke et al. 2004). After transformation strain identity was confirmed by colony

PCR using gene specific test primers and internal primer (Nat_ctrl_Hb) from the insert (Lõoke et al. 2011). All the details regarding strains, plasmids and primers are given in the Table 1. All primers were synthesised by Sigma Aldrich.

Growth conditions and media

All starvation experiments were conducted in synthetic dextrose (SD) media (Verduyn et al. 1992) with all necessary auxotrophic agents added in surplus (Pronk 2002). For purine starvation, synthetic media without adenine was used.

Growth rate analyses were performed in a 96 well multimode reader TECAN Infinite M200 PRO. *S. cerevisiae* cells were grown in synthetic media containing glucose, washed three times with distilled water and resuspended in the synthetic media with all combinations of ammonia/proline and glucose/galactose as N and C sources.

To assess adenine starvation effect on desiccation tolerance, cells were grown in full SD media up to exponential phase (OD_{600} less than 1), washed with distilled water, and re-suspended to $OD_{600} = 0.5$ in SD media without adenine, but with all other broth components added. The yeasts were incubated for 4 h in a rotary shaker (140 rpm, 30 °C) and then desiccated.

Desiccation tolerance assay

Desiccation tolerance was assayed by estimating CFU mL⁻¹, before desiccation and after dehydration. One millilitre of culture at $OD_{600} = 1$ was washed with distilled water twice, diluted serially and spotted on YPD (yeast extract 1%, bactopeptone 2%, glucose 2%, agar 2%) plates. The

Table 1. Yeast strain, plasmids and primers used in the present study

	Name	Description/sequence/	Source
Yeast strain	WT	CEN.PK2-D <i>MATα leu2-3/112 ura3-52 trp1-289 his3Δ1 MAL2-8c SUC2 Δade8</i>	Kokina et al. 2014
Plasmid	pYM46	PCR template for C-terminal myc tag plus 7 His residues: marker pAgTEF-kanMX-tAgTEF, selectable phenotype: G418 resistance	Janke et al. 2004, EUROSCARF
Primers	MSN4_S2	CTTGTCTTGCTTTATTGCTTTGACCTTATTTC TTTCAATCGATGAATTGAGCTCG	This study
	MSN4_S3	GCATTCCAGACGCACTGAGCACTTGAAAAGGCATA TAAGATCGTACGCTGCAGGTCGA	This study
	MSN2_S3	GAAATTAGTAGAAGCGATAATTGTCGCAACAC ATCAAGCGTACGCTGCAGGTCGA	This study
	MSN2_S2	TGAAGAAAGATCTATCGAATTAAAAAAATGGGT CTATTAATCGATGAATTGAGCT	This study
	RIM15_S2	CAGTTATTTTTTAATTATCTTATCTTAAATT TATCAATCGATGAATTGAGTCG	This study
	RIM15_S3	CAGGAGGCCAACCAACAGTAGAGCTTTGACGAT GTTTAGCGTACGCTGCAGGTCGA	This study
	MSN4_test_F01	AGAAGGCATTACAGACCGAGT	This study
	RIM15_test_F01	CCAATTGTGGCCATAACAAA	This study
Jli_MSN2-t01	CCATTATCGCCTGCATCATCAT	This study	
NAT-HgB_ctrl	ACGAGGCAAGCTAACAGATCT	This study	

remaining cell suspension was centrifuged, and the pellet was left to desiccate for 6 h at 30 °C in a desiccator, then diluted in distilled water in room temperature. Rehydrated cell solution was thoroughly mixed, serially diluted and spotted on YPD plates. The viability (%) was calculated by dividing the number of CFU mL⁻¹ after and before desiccation, similarly to Calahan et al. (2011).

Statistics

All experiments were performed in triplicates, error bars represent standard deviations. Asterix is used where $p < 0.05$.

Results and discussion

Adenine starvation halts cell proliferation

At first we determined effect of adenine starvation on the wildtype cells growth dynamics. Results are shown in the Fig. 1.

S. cerevisiae wildtype strain used in this study has multiple auxotrophies. It is auxotroph for histidine, adenine, tryptophan, leucine and uracil. As a well characterised control for yeast cell auxotrophic starvation, we added uracil deficient media and observed the growth pattern in this medium. When cultivated in adenine or uracil deficient media, auxotrophic starvation led to halt of cell proliferation, as cells lack building blocks for further growth. Previously we have observed that optical density alone is not a reliable measurement of actual cell number in the case of adenine-starved cells. While starving for adenine, *S. cerevisiae* cells tend to increase in volume and therefore cell number per mL in unit of absorbance

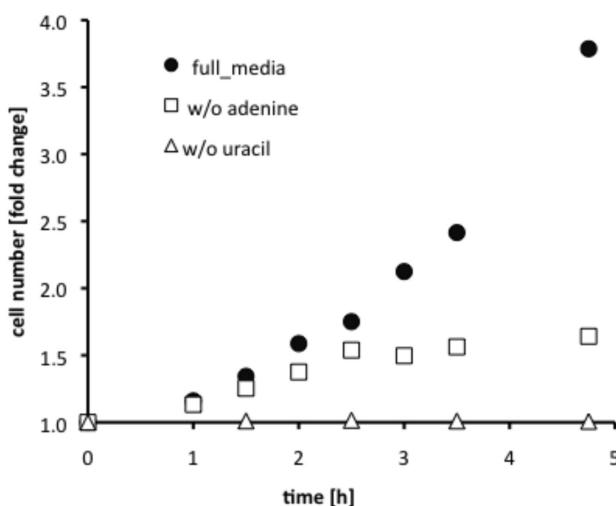


Fig. 1. *S. cerevisiae* wildtype growth dynamics in full SD media and in the SD media without adenine or uracil supplement (w/o adenine or w/o uracil). Growth dynamics were registered with a Z™ Coulter counter® (Beckman Coulter). It is depicted as fold change (cell number of the each time point normalised to initial cell number). Data points are average from independent triplicates; standard deviation is less than 4%, error bars not visible.

value is affected (Kokina et al. 2014). To be sure that we observed only halt of cell proliferation rather than increase in cell volume, we used direct cell counts via particle counter, not spectrophotometrical OD measurements. To compare cultivations with different initial cell numbers, we normalised all cell number per mL against initial cell number (see Fig. 1).

Cells cultivated in adenine free, synthetic dextrose media cease to multiply later than in the case of uracil starvation. Based on our data (Fig. 1), we assumed that adenine starvation sets in 2 to 3 h after a shift to starvation media, while in the case of uracil starvation, it set in immediately after media shift. Therefore, to induce a “adenine starvation phenotype”, we cultivated wildtype cells in adenine deficient media for 4 h.

Msn2/4p and Rim15p C-end truncation affects cell growth

Transcription factors were truncated by transforming yeast with the linear PCR fragment containing flanking homologous sequences of the respective transcription factor and 6xHis tag and G418 marker in between (Janke et al. 2004). When inserting fragments, the last 100 bp of coding sequence were removed. The last 50 amino acids of MSN2/4p code for DNA binding Zn finger domain and are responsible for binding to target STRE sequences (Estruch, Carlson 1993). Rim15p contains REC domain in its C-end; it seems to be involved in autophosphorylation and dimerisation activities (Wanke et al. 2005).

We tested growth of truncated strains in synthetic media with varying carbon and nitrogen sources. We chose glucose and galactose as repressive and derepressive examples of carbon sources and ammonium sulphate as example of repressive and proline as non-repressive nitrogen source (Gancedo 1998; Cooper 2002). As a result, we observed different growth patterns when strains were grown under different C or N sources. This reflects divergent functions that these factors have in adopting cell growth in the presence of different (repressive or derepressive) carbon or nitrogen sources, see Fig. 2. Previously we had confirmed identity of our constructs by colony PCR (Lõöke et al. 2011); these media shift studies served as functional confirmation of successful gene truncation and effect on the cell signalling system.

In synthetic media with glucose and ammonia as carbon and nitrogen sources, the Msn2p C-end truncated strain exhibited similar specific growth rate (μ) as the wildtype. Strains with Msn4p, and Rim15p C-end truncated grew 25% slower (see Fig. 2), 0.4 h⁻¹ for the wildtype and *msn2*, 0.28 h⁻¹ and 0.26 h⁻¹ for *msn4* and *rim15*, respectively. Similar effects of Msn2 functional impairments on strain growth have been demonstrated previously by Estruch and Carlson (1993). In both cases, C-end specific truncation (our results) and *msn2/4* partial knockouts (Estruch, Carlson 1993) resulted in hampered growth of the strains on galactose as the main carbon source.

In all carbon-nitrogen source combinations, the

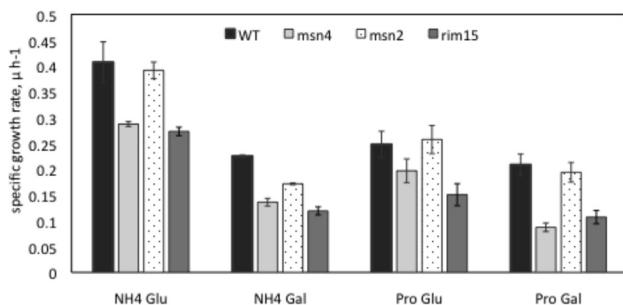


Fig. 2. Growth rate ($\mu\text{ h}^{-1}$) of the wildtype (WT) strain and its corresponding strains with C-end truncated *msn2*, *msn4*, *rim15* mutants cultivated in SD+ media with different carbon (Glu, glucose; Gal, galactose) and nitrogen (NH₄, ammonium; Pro, proline) sources. Each bar depicts the mean of four independent cultivations. Error bars represent standard errors.

Rim15p C-end truncated strain grew slower and exhibited a comparatively long lag phase. Interestingly, low growth speed and extended lag phase have been observed with sake yeast possessing a frame shift mutation in the Rim15p C-end and lacking 75 amino acids of the C-end (Watanabe et al. 2012).

The nitrogen source shift affected Msn2/4p C-end truncated strains in the same way as the wildtype. Derepressing the nitrogen source together with derepressing the carbon source decreased growth of all strains, since carbon and nitrogen metaboslims share common metabolites, like α -ketoglutarate (Cox et al. 2002; Tate, Cooper 2013). The results showed that the introduced changes are more responsive to changes in the carbon, not nitrogen source.

*Msn2/4 and Rim15 C-end truncation affects *S. cerevisiae* cell desiccation tolerance after adenine starvation*

Desiccation tolerance is a typical feature of *S. cerevisiae*, which is often used for biotechnological purposes (like preparation of dried yeast for bread, beer and wine production). To survive desiccation, cells should be ready for a number of stresses (oxidative, salt, heat, etc.; review in Dupont et al. 2014). It is also known that condition of a cell before desiccation directly correlates to survival of desiccation tolerance. (Welsh et al. 2013). Here we use desiccation “stress” as a “environmental stress” to determine the activity state of ESR.

We attempted to determine if transcription factors Msn2/4p or their upstream kinase Rim15p have any role in adenine starvation induced desiccation tolerance in *S. cerevisiae*. We grew wildtype and derived strains with C-end truncated Msn2/4p or Rim15p till OD₆₀₀ approx 1 or less, harvested the cells, washed them, and resuspended them in SD complete broth or SD lacking adenine with final OD₆₀₀ = 0.5. After cultivation in shake flasks on rotary shaker for 4 h, 30 °C, we harvested cells from all the shake flasks, desiccated and spotted them on solid YPD agar and

counted colonies. Viability was calculated as proportion of culture CFU after desiccation against CFU before desiccation. Desiccation tolerance results are shown in Fig. 3.

After adenine starvation, we obtained similar desiccation tolerance results for wildtype as published previously (Kokina et al. 2014). We observed a huge (more than 1000 times) increase in desiccation tolerance in the adenine starved cells when compared to desiccation tolerance of rapidly growing wildtype cells.

Desiccation tolerance of all C-end truncated strains were statistically significantly different from the wildtype. In the case of strains with C-end truncated Msn2/4p, desiccation tolerance after adenine starvation was lower than in wildtype cells. Desiccation is rather complex treatment, which includes elements of oxidative, heat shock and osmotic stresses (Franca et al. 2005). Therefore, functional impairment of transcription factors responding to all those stresses (Msn2p and Msn4p) will inevitably lead to lowered tolerance. However, drop of the viability in these strains was within the same order of magnitude. Since our spot test allowed us to estimate population viability from 100% down to 0.00001%, therefore a “significant signal” when using such a rough metric indicated changes in orders of magnitudes and not changes within the same order of magnitude. Calahan et al. (2011) observed a drop in desiccation tolerance in *msn2* and *msn4* knockouts within the same order of magnitude in saturated cultures, similarly to our C-end *MSN2/4* truncations.

Interestingly, Rim15p C-end truncation led to increase in desiccation tolerance in exponentially growing culture (SD media with all necessary supplements). It reached 0.03%, which is approximately 50 times more than typical desiccation tolerance of the wildtype and other strains when grown in full media. In comparison, the Rim15p

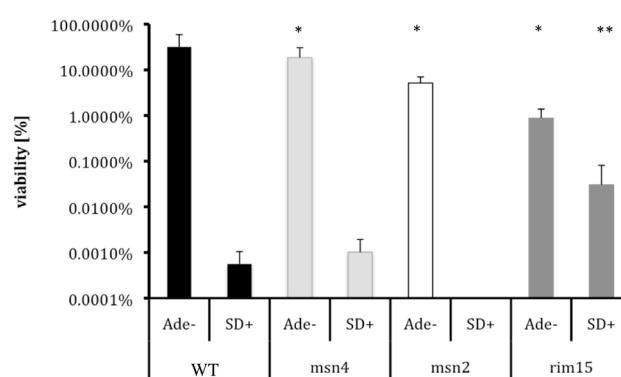


Fig. 3. Desiccation tolerance of wildtype (WT) and its derivative strains with C-end truncated *msn2*, *msn4* or *rim15*, after 4 h cultivation in full SD media (SD+) or SD media without adenine (Ade-). Desiccation was performed at 30 °C in a desiccator. Each bar represents the mean of three independent samples, error bars represent standard deviations. Stars depict significant differences ($p < 0.05$) between wildtype and engineered strain adenine starvation treatment (*) and full media (**).

C-end truncated strain desiccation tolerance after adenine starvation increased just 30, not 1000 or more times, when compared to wildtype.

Truncation of Rim15p led to a more severe desiccation tolerance decline than in the *msn2/4* double knockout (Calahan, et al. 2011). This indicates that there are other elements of Environmental Stress Response system responsible for adenine starvation specific, stress tolerant phenotype formulation, except besides *MSN4* and *MSN2*.

Sake yeasts form a compact *S. cerevisiae* strain subgroup, where at least seven strains have C-end truncated versions of Rim15p (Liti et al. 2009; Wang 2012; Watanabe 2012). We observed that the strain with Rim15p C-end truncation had increased desiccation tolerance in its exponential phase. While several sake yeast strains ferment glucose to ethanol rapidly, they are characterised for low ethanol and heat stress tolerance. This has been linked to defects in Msn2/4p signalling (Watanabe 2011). Also, these strains enter quiescent state with low efficiency (Urbanczyk, 2011). Recently, genetic defects in the Msn2/Msn4 upstream regulator *RIM15* have been found. When transforming cells with the “correct” version of *RIM15*, growth, heat and ethanol tolerance defects were complemented (Watanabe 2012).

Our results allow us to speculate that the Rim15p C-end truncated version might have phenotypic advantage during sake production. We presume that improved ethanol production together with comparatively “increased” desiccation tolerance in the exponential phase could be a phenotype being unintentionally selected throughout the rich history of sake production (Kitigaki, Kitogoto 2013).

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