UNIVERSITY OF TARTU
Faculty of Science and Technology
Institute of Technology

Nadezhda Chulkova

Characterization of *Saccharomyces cerevisiae* and its cAMP dependent protein kinase A deletions in response to saline stress

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

Supervisor(s):
Research Fellow, Rahul Kumar, Ph.D.
Senior scientist, Petri-Jaan Lahtvee, Ph.D.

Tartu 2020

Abstract:

Protein kinase A (PKA) activity, dependent on intracellular concentration of cAMP, has a significant influence on cellular stress response in the budding yeast *Saccharomyces cerevisiae*. PKA protein consists of three catalytic subunits Tpk1p, Tpk2p and Tpk3p, and one regulatory subunit Bcy1p. The catalytic subunits phosphorylate transcription factors (TFs) Msn2p/Msn4p. Msn2p and Msn4p are essential for the activation of many of the genes involved in the stress response in yeast. These TFs also regulate the activity of Yak1p, which functions in the glucose-signaling system and impacts growth upon nutrient deprivation in yeast. This thesis focuses on the physiological characterization of saline stress response of *Saccharomyces cerevisiae* (W303 background) and its two PKA-deletion strains, i.e., Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1* and Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4* deletions, where deletions of Δ*yak1* and Δ*msn2* Δ*msn4* made the triple Tpk1p subunit deletions containing stains viable. The saline stress response in Δ*yak1* and Δ*msn2* Δ*msn4* was also determined.

Conclusions: Deletion of *MSN2/MSN4* or *YAK1* allows a similar growth as the reference strain in otherwise non-viable triple Δ*tpk* deletion strain. Salt stress has a negative impact (40-70%) on growth rates of all the strains in the study. The efficient production of glycerol under salt stress likely suggests that the HOG pathway is not directly dependent on PKA or Msn2p/Msn4p or Yak1p.

Keywords:

Saccharomyces cerevisiae, cAMP-dependent protein kinase A (PKA), Takashi protein kinase (Tpk1,2,3), Ras-cAMP pathway, osmotic stress, saline stress

CERCS:

T490 Biotechnology

Saccharomyces cerevisiae ja tema cAMP sõltuva proteiinkinaas A deletiooni mutantide iseloomustamine soola stressi tingimustes

Lühikokkuvõte:

Proteiinkinaasil A (PKA) on oluline roll pärmi *Saccharomyces cerevisiae* stressivastuses. PKA aktiivsus sõltub cAMP rakusisesest kontsentratsioonist. PKA valk koosneb kolmest katalüütilisest subühikust Tpk1p, Tpk2p ja Tpk3p ning ühest regulatoorsest subühikust

Bcy1p. Katalüütilised subühikud fosforüleerivad transkriptsioonifaktoreid (TF) Msn2p/Msn4p. Msn2p ja Msn4p on olulised mitmete stressivastustega seotud geenide aktiveerimisel. Need TFid reguleerivad ka Yak1p aktiivsust. Yak1p osaleb glükoosi signaliseerimise süsteemis ja mõjutab pärmi kasvu toitainete puudumisel. Käesolev lõputöö keskendub soolast tingitud stressivastuse füsioloogilisele kirjeldamisele pärmis *Saccharomyces cerevisiae* (W303 taust) ja tema kahes PKA deletsiooniga tüves, s.t. Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1* ja Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4.* Δ*yak1* ja Δ*msn2* Δ*msn4* deletsioonid muutsid tüved, mis sisaldasid kolmik Tpk1p subühiku deletsioone, eluvõimeliseks. Soola-stressi vastus tuvastati ka tüvedes Δ*yak1* ja Δ*msn2* Δ*msn4.*

Kokkuvõte: MSN2/MSN4 ja YAK1 deletsioonid võimaldavad pärmil referentstüvele sarnast kasvu isegi tavaliselt mitteelujõulise kolmik Δtpk deletsiooniga tüve puhul. Soolastress mõjutab kõikide antud töös uuritud tüvede kasvu negatiivselt (40-70%). Efektiivne glütserooli tootmine soolastressi korral näitab, et HOG-rada ei sõltu otseselt PKA-st, transkriptsioonifaktoritest Msn2p/Msn4p ega kinaasist Yak1p.

Võtmesõnad:

Saccharomyces cerevisiae, cAMP-sõltuv proteiinkinaas A (PKA), Takashi proteiinkinaas (Tpk1,2,3), Ras-cAMP rada, osmootne stress, soolastress

CERCS:

T490 Biotehnoloogia

TABLE OF CONTENTS

TERMS, ABBREVIATIONS AND NOTATIONS

YPD - Yeast-extract peptone dextrose

PKA –Protein kinase A.

Msn2p, Msn4p – multicopy suppressor of SNF1 mutation. Paralog proteins, stress-responsive transcription factors.

Yak1p – yet another kinase 1 (Garrett & Broach, 1989). Inhibits cell growth in response to glucose depletion.

Tpk1p, Tpk2p, Tpk3p – Takashi's protein kinases, catalytic subunits of protein kinase A.

Bcy1p – bypass of CYclic-AMP requirement, a regulatory subunit of protein kinase A.

Rim15p – regulator of IME2, protein kinase, phosphorylates Msn2p and Msn4p.

Hog1-MAP pathway the high-osmolarity glycerol (HOG) and mitogen-activated protein (MAP) kinase cascade pathway.

cAMP – cyclic adenosine monophosphate, a second messenger used for intracellular signal transduction.

HPLC – high-performance liquid chromatography

gDCW – grams dry cellular weight

INTRODUCTION

During evolution, organisms have acquired the ability to adapt to environmental changes. The environmental stress response (ESR) function allows the maintenance of vital cellular processes within an organism while it is under stress conditions (Gasch et al., 2000). There are various stress factors, such as heat-shock, saline stress, oxidative stress, and many other types as well. This thesis focuses on studying saline stress, which has two major effects: a hypoosmotic shock (osmolarity of the environment is much higher than within a cell) and cationic toxicity (K+ ions are being replaced with Na+) (Hohmann, 2002). The thesis studies saline stress response of the baker's yeast *Saccharomyces cerevisiae*, which is a common model for investigation of fundamental processes in eukaryotic organisms. Yeast stress response (including saline stress response) is mostly controlled by the expression of the genes that are dependent on Msn2p and Msn4p transcription factors (TFs) activity (Gasch et al., 2000). These TFs are involved in the cAMP-dependent protein kinase A (PKA) metabolic pathway in yeast. PKA is a heterotetramer, which consists of two regulatory subunits (Bcy1p) and two catalytic subunits (it can be Tpk1p, Tpk2p, Tpk3p). The PKA activity is dependent on intracellular cAMP (a second messenger) levels, which is accumulated when the glucose is present in the environment. Accumulated cAMP binds to PKA regulatory subunit Bcy1p, leading PKA catalytic subunits Tpk1p, Tpk2p, Tpk3p to phosphorylate Msn2p and Msn4p transcription factors. Phosphorylated Msn2p and Msn4p are exported to the cytoplasm and, this prevents activation of Yak1p kinase, which is negatively regulated by PKA and is involved in a glucose-sensing system that impacts growth upon nutrient depletion (Lee et al., 2008).

This thesis characterized the physiology of the following strains in the presence or absence of PKA activity, namely, *S. cerevisiae W303*, Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4*, Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1*, Δ*msn2* Δ*msn4*, and Δ*yak1*. Briefly, the study design is described. Firstly, reproducible plate reader experiments were performed to investigate tolerance for different NaCl concentrations (1.4 M, 1.6 M, 1.8 M, or 2.0 M) and their impact was monitored by determining the changes in the specific growth rates of deletion strains compared with the reference strain. The growth characterization in response to saline tolerance allowed the identification of a viable saline concentration, i.e. NaCl (1.6 M) for performing further experiments in shake flasks. A chemically defined culture medium with 1.0% glucose was used for physiology characterization without NaCl in the control experiments and with NaCl (1.6 M) in the saline stress experiments. The physiological differences between the control and

saline stress conditions were determined based on the changes in specific growth rate, length of lag phase, specific glucose consumption rate, and specific production rates of byproducts.

1 LITERATURE REVIEW

1.1 *Saccharomyces cerevisiae* – a model unicellular eukaryotic organism

Yeast – a unicellular eukaryotic fungus - was most likely the first microorganism used by humans to process food and produce alcohol. *Saccharomyces cerevisiae* (often called brewer's or baker's yeast') is the most common yeast used, both, in industry and for scientific research. *S. cerevisiae* and other related fungi are known for their ability to ferment glucose (Bisschops, 2014), a process widely used in the food industry. At the same time, *S. cerevisiae* can be used as a cell factory for biological production of different chemical compounds, starting from natively produced ethanol (used as biofuel) to the proteins, used in the food industry and pharmaceuticals, e.g. human serum albumin or hepatitis vaccines (Borodina & Nielsen, 2014). An extensive understanding of metabolism and regulation of yeast allows us to design various efficient strategies for the production of interesting compounds.

Moreover, during the last decades yeast has also become a popular organism for investigating intercellular mechanisms. While studying *S. cerevisiae,* Leland Hartwell was the first to describe a set of genes that control the cell division process (Hartwell et al., 1973). The discovery of the subunits controlling cell cycle became a fundamental research topic, that contributed to further investigation of the processes occurring in living organisms and was awarded the Nobel prize in 2001. Moreover, with the advent of high-throughput genomics technologies, the *S. cerevisiae* genome was the first fully sequenced eukaryote, which further accelerated the studies of this organism (Goffeau et al., 1996). Functional genomics studies allowed the determination of gene functions in yeast and other organisms. Currently, all *S. cerevisiae* genes together with its function have been identified (Costanzo et al., 2016), which has opened prospects for further research and development of applications using yeast.

1.2 Stress response pathways in *S. cerevisiae*

Due to growth, all cells experience changes in their extracellular environment. Also, a lack of adaptation to a new environment can be deleterious to a cell (Ivorra et al., 1999). Thus, the survival of an organism depends a lot on their ability to adapt to environmental changes. During evolution, the mechanism of adaptation - the environmental stress response (ESR) - has been developed to protect and maintain vital processes of yeast in response to various signs of potential changes in the environment. The ESR is driven both by an up-regulation

of genes dependent on transcription factors Msn2p and Msn4p activity, and down-regulation of the genes, associated with ribosome transcription and translation (Gasch et al., 2000). Investigation of stress response to different environmental changes has an important role in understanding cellular adaptation mechanisms and is relevant for the investigation undertaken in this thesis.

1.2.1 Commonly encountered stress factors by *S. cerevisiae*

Any environmental factor which negatively affects yeast growth is defined as a stress factor (Ivorra et al., 1999). Audrey P. Gasch described *S. cerevisiae* genomic expression programs in response to 13 types of stress conditions (Gasch et al., 2000). The following table label s and classifies the most common and wide-spread types of yeast stress (Table 1) (Ivorra et al., 1999).

Table 1. Causes and resulting stresses for *S. cerevisiae.*

Stress type	Condition
Heat shock	Environment temperature is above the optimal temperature
Hypoosmotic shock	Significant decrease in the osmolarity of environment fluid
Hyperosmotic shock	Significant increase in the osmolarity of environment fluid
Ethanol	Presence of ethanol in the environment
Low pH (acetic acid)	Non-optimal pH of the environment
Oxidative stress	Exposure to reactive oxygen species (H_2O_2)

1.2.2 Reasons of osmotic stress

Control of water content (osmoregulation) is one of the fundamental biological processes, occurring within a cell. It is determined by relative water concentration within and outside the cell. There are systems within a cell that make it possible to survive and adapt to the rapid shifts in water activity (Hohmann, 2002). Yeast cell volume changes in response to osmotic challenges: it increases during hypotonic (osmotic downshift) stress and decreases while hypertonic (osmotic upshift) stress condition. During an osmotic shift, the cell should react immediately as water loss and uptake occur very fast (Blomberg et al., 1992). Under osmotic-stress Hog1 (high osmolarity glycerol response)-MAP kinase pathway activates,

leading to glycerol accumulation, which plays the central role in osmoadaptation. Glycerol is the most common osmolyte in yeast, that serves as a protectant for proliferating cells, especially during extreme kinds of osmotic stress (freezing and desiccation) (Hohmann, 2002). At the same time, MAP kinase plays an important role in stress response regulation (including osmoadaptation) in yeast and other eukaryotes (Waskiewicz & Cooper, 1995).

There are plenty of conditions that can cause osmotic stress:

- Two extreme forms of osmotic stress
    - Freezing – extremely cold temperature, leading to the change of intercellular water structure (as water is frozen at the temperatures below 0°). That can cause irreversible damage to the cell.
    - Desiccation – an extreme decrease of intercellular water level (dehydration). Evaporation of water from the environment surrounding the cell increases the external concentration of the solute, which leads to hyperosmotic stress and increased salinity (Calahan et al., 2011).
- Ethanol. A high concentration of the ethanol in medium affects the hydration of the intercellular molecules (Hallsworth, 1998).
- Salt stress. Saline solutions (e.g. containing NaCl) have increased osmolarity, leading to hyperosmotic stress in the cell (Hohmann, 2002).
- The presence of other solutions (e.g. sorbitol, mannitol, PEG-6000) in the environment can also cause osmotic stress in the yeast cells.

1.2.3 Relationship between osmotic stress and salt stress

The effect of the salt stress on yeast cells implies both specific cationic toxicity and osmotic stress. Firstly, some ions - including Na^+ can replace intercellular K^+ – and inhibit metabolic pathways, thus, can be toxic (Serrano et al., 1997). In the case of salt stress, cellular energy is invested for the accumulation of the intercellular K^+ and maintenance of low Na^+ level, as potassium is crucial for many physiological functions (e.g. protein synthesis and regulation of the cell volume) (Hohmann, 2002).

Secondly, the saline shock causes the increase of intercellular osmolarity, leading to the induction of the genes, involved in osmotic stress response (Hohmann, 2002). Many of those genes are activated by transcription factors Msn2p and Msn4p (one of the targets of the current study).

1.3 Ras-cAMP pathway in *S. cerevisiae*

1.3.1 Function and composition of cAMP-dependent protein kinase A (PKA)

Nutrient signaling networks are responsible for a successful adaptation to changes in the environment. In yeast, various intracellular processes, dependent on the quality and amount of carbon source, are associated with different signaling pathways.

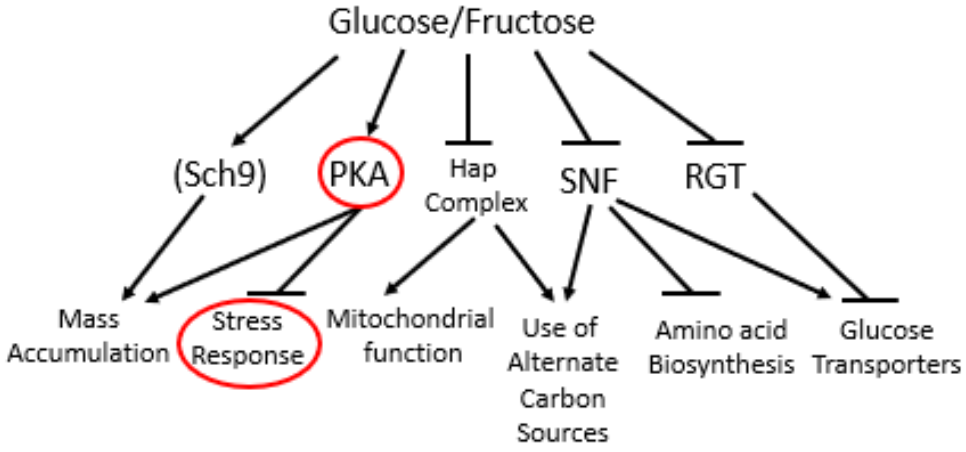


Figure 1. Carbon source-dependent yeast signaling pathways (source: Broach, 2012)

cAMP-dependent protein kinase A (PKA) pathway is known to influence cell growth and cellular stress response accordingly to glucose level (Broach, 2012). The exposure to glucose immediately leads to an increase in the concentration of internal cAMP – a second messenger, required for intracellular signal transduction. PKA is a heterotetramer composed of two regulatory subunits, encoded by *BCY1*, and two catalytic subunits, encoded by three related genes – *TPK1*, *TPK2*, *TPK3*. Regulatory subunit (Bcy1p) is required in such situations as growth on nonfermentable carbon sources or switch to the stationary phase (Werner-Washburne, 1991): it inhibits PKA activity in response to glucose starvation, (in this case cAMP is not being accumulated within a cell). In the presence of glucose, the cell accumulates cAMP, which binds to Bcy1, preventing its inhibitory activity on the catalytic subunits (Broach, 2012) **(Figure 2)**.

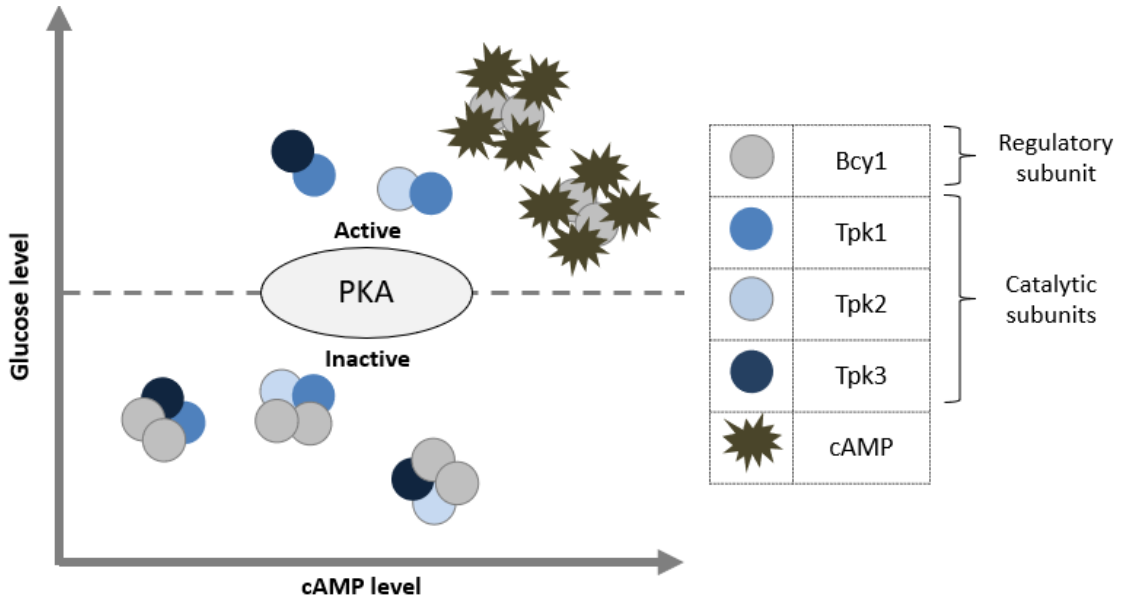


Figure 2. PKA conformation. When glucose is present in the environment, cAMP binds to the regulatory subunit of PKA, thus, PKA catalytic subunits are not inhibited any more.

1.3.2 The role of PKA in *MSN2/4* and *YAK1* activation

PKA can phosphorylate Msn2p and Msn4p. Msn2p and Msn4p transcription factors are required for the expression of the genes, containing STRE (stress response element) on their promoter (Lee et al., 2008). One of those genes encodes protein kinase Yak1p, which is known by its ability to inhibit cell growth in response to depletion of glucose from the environment (Garrett et al., 1991). PKA dependent phosphorylation of Msn2p and Msn4p blocks their translocation from cytoplasm to nucleus and thereby reducing YAK1 transcription.

YAK1 expression is negatively correlated with PKA activity and its activity is increased in the PKA-depleted strains (Garrett et al., 1991). Under the low glucose levels, PKA is not active and Yak1p becomes active where it inhibits cell growth and stimulates the stress response by activation of Msn2p in yeast (Figure 3)(Lee et al., 2008).

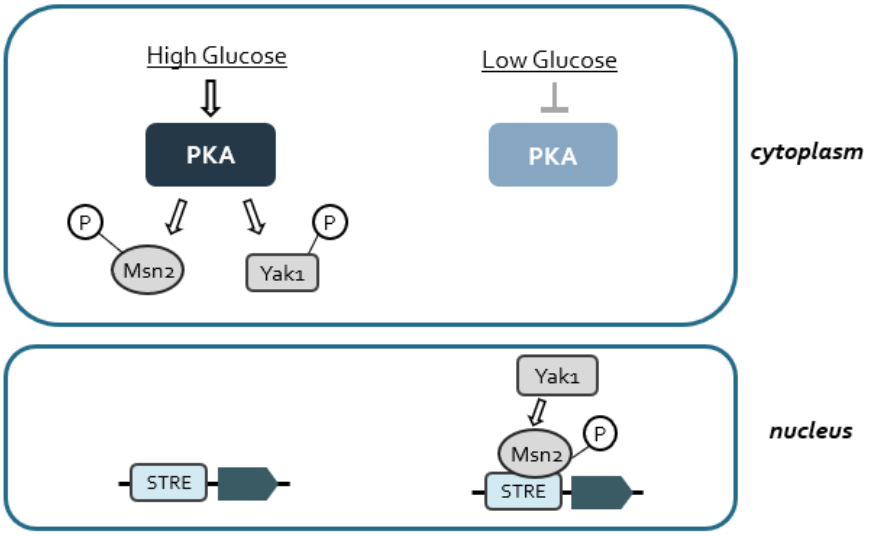


Figure 3. Yak1-dependent regulation of Msn2p. Under the high levels of glucose, protein kinase A phosphorylate Msn2p and Msn4p, inhibiting their nuclear localization. Otherwise, Msn2p and Msn4p are translocated to the nucleus and induce expression of the genes that contain STRE (stress response element) in the promoter (source: Lee et al., 2008)

1.3.3 The role of *TPK1,2,3*, *MSN2/4* and *YAK1* gene deletion

Any of three PKA catalytic subunits is enough to maintain the viability of the cell, while Δ*tpk1* Δ*tpk2* Δ*tpk3* deletion strain (null mutant) is not viable (Broach, 1989). PKA negatively regulates Yak1p protein kinase and interacts with Msn2p and Msn4p proteins to control cell growth (Peter Lee et al., 2008). Some side effects of PKA functional loss can be counteracted by the deletion of Yak1p or Msn2p and Msn4p in yeast (Livas et al., 2011). Such multiple mutants can be used to investigate PKA-independent stress regulations in yeast, as strains lacking Msn2p/Msn4p or Yak1p do not have an obligatory requirement of PKA for the growth (Livas et al., 2011).

The transcription factors Msn2p/Msn4p are also known to activate gene expression in response to multiple stresses, including osmotic stress (Smith et al., 1998). In the absence of Msn2p and Msn4p, there is no transcription of the genes involved in some stress responses (Livas et al., 2011). Mutant strains, lacking Msn2p and Msn4p, are susceptible to different forms of stress (heat, osmotic stress), failing to accumulate stress-regulated signals (Hohmann, 2002). In 2000, Audrey P. Gasch et al. studied almost 300 genes whose expression was positively dependent upon different stress conditions, and among them, 180 genes were affected by deletion of the genes encoding Msn2p and Msn4p or, vice versa by its overexpression (Gasch et al., 2000). It shows that Msn2p and Msn4p control the expression of most of the genes involved in stress responses.

2 THE AIMS OF THE THESIS

Physiological characterization of *Saccharomyces cerevisiae* in response to saline stress and deletion of cAMP-dependent protein kinase A.

1. To investigate different saline concentrations to determine viable stress tolerance by studying effects on the growth of *S. cerevisiae* W303 and its deletion strains.
2. To characterize the stress response physiology of the reference *S. cerevisiae W303* and the strains lacking protein kinase A activity in the presence of a viable salt concentration.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Yeast strains:

The strains used in the present study were constructed by Livas et al., 2011 and were kindly acquired for the present research purpose by the Petri-Jaan Lahtvee lab (Table 2).

Table 2. The list of *S. cerevisiae W303* strains used in this thesis research.

Strain	Genetic background
567	**W303 wild-type**
563	**Δ*msn2* Δ*msn4***
569	**Δ*tpk1* Δ*tpk2*Δ *tpk3* Δ*msn2* Δ*msn4***
571	**Δ*tpk1* Δ*tpk2*Δ *tpk3* Δ*yak1***
573	**Δ*yak1***

3.1.2 Media

In the study, at different steps, different culture mediums were utilized. For pre-culture, 0.5 L of YPD (yeast peptone dextrose) media was prepared. For the plate reader experiment 0.5 L of the Delft minimal media containing 0.5% glucose was prepared as previously reported (Kumar & Lahtvee, 2020). Minimal media pH was adjusted to 6.0 with KOH (potassium hydroxide). Briefly, all compounds were mixed in distilled water and autoclaved 20 min under 121°C. Glucose solution was autoclaved in a separate bottle to avoid its interaction with other compounds and further conversion to another molecule. The trace metals and vitamin solutions were filter sterilized and added to the culture medium under aseptic conditions after autoclavation and at room temperature.

During the screening experiment in the microplate reader experiment, this media was supplemented with various salt concentrations (1.4 M, 1.6 M, 1.8 M, 2.0 M of NaCl). For the shake flask experiment, the Delft minimal medium containing 1% glucose was used as a

control and it was supplemented with 1.6 M NaCl for the study of saline stress response (Table 3).

Table 3. Medium compositions used in the current study.

Media		Composition
YPD (yeast peptone dextrose)		20 g/L peptone (Formedium) 10 g/L Micro granulated Yeast Extract (Formedium) 20 g/L D-Glucose anhydrous
Delft minimal medium		7.5 g/L $(NH_4)_2SO_4$ 3.5 g/L KH_2PO_4 0.75 g/L $MgSO_4 \cdot 7H_2O$ 1ml/L mineral solution 1 ml/L vitamin solution.
Delft minimal medium	+0.5% glucose	5 g/L glucose
	+1.0% glucose	10 g/L glucose
	+1.4 M NaCl	81,9 g/L NaCl
	+1.6 M NaCl	93,6 g/L NaCl
	+1.8 M NaCl	105,3 g/L NaCl
	+2.0 M NaCl	117 g/L NaCl

3.1.3 Plate reader experiment:

Yeast was grown at 30° in nutrient rich YPD media overnight (~18 hours). It was followed by washing with PBS (phosphate-buffered saline) and transferred to 0.5% glucose-containing minimal media and collected after 6 hours of cultivation for the future experiment. Yeast was diluted to get OD600nm ~0.1. The NaCl concentrations used in the study were 1.4 M, 1.6 M, 1.8 M, and 2.0 M. The plate reader experiments were performed in the 96-well plates were using 200 µl of the final sample volume with starting inoculum at OD600nm ~0.1 and increase in OD was monitored online. The experiments were carried out at 30°C and continuously shook for 42 hours in the aerobic environment. The OD measurements were taken every 30 minutes for triplicates.

3.1.4 Shake flask experiments

Yeast was grown at 30°C in the nutrient rich YPD media overnight, then transferred to 1% glucose-containing minimal media and collected after 6 hours of growth for future experiments.

During the experiment, yeast cells were grown in 125 ml flasks in 15 ml of media. The cultures were placed in the shaker at 30° with continuous stirring at 200 rpm. During the exponential phase samples were collected for HPLC and OD600 every 2 hours, additional samples were collected during the stationary phase for duplicates.

3.1.5 Optical density measurement

For OD measurement, Hitachi U-1800 spectrophotometer was used. Measurements were taken at 600 nm wavelength (OD600 0.1 corresponds to approximately $1*10^{-6}$ cells ml^{-1}). For blank measurements, 1 mL of 0.5% glucose delft minimal media was used.

3.1.6 Specific growth rate calculation

The specific growth rate for plate reader and shake flask experiments data was calculated using the formula described below:

$$\mu = \frac{\ln \frac{x2}{x1}}{\Delta t},$$

where,

μ - growth rate
x_1 – number of the cells (in this case, OD600) in the first time point
x_2 – number of the cells in the second time point
Δt – the time between two measurements (hours)

3.1.7 Specific production and consumption rates calculation

For yeast, OD600 1.0 is equal to ~$3 * 10^{10}$ cells/liter (Milo, 2010). The weight of the haploid cell is ~$15*10^{-12}$ grams (Milo, 2010). Thus, OD600 1.0 refers to ~0.45 g/L cells. To calculate the biomass (X), the following formula was applied:

$$X = OD600 * 0.45 \ (gDCW/l)$$

The yield (Y) was calculated with this formula:

$$Y = \frac{X}{g\ substrate\ utilized} \left(\frac{g\ biomass}{g\ substrare}\right)$$

The formula below was used to calculate the specific substrate consumption and production rates:

$$r = Y * \mu \left(\frac{g}{gDCW * h}\right)$$

3.1.8 Extracellular metabolome quantification

3.1.8.1 Standards and sample preparation.

During the shake flask experiment, 500 µl of samples were collected into Eppendorf tubes. Samples were centrifuged 5 min at 5500 rpm. After this step, they were moved to a new eppendorf tube and centrifuged for 5 min at the maximum speed at 14800 rpm. The supernatant was collected to a new eppendorf tube and stored (where) until analysis. Samples, containing high concentrations of NaCl, were diluted 4x with distilled water. 175 µl of the final sample was added to a glass vial for further HPLC analysis (Figure 4).

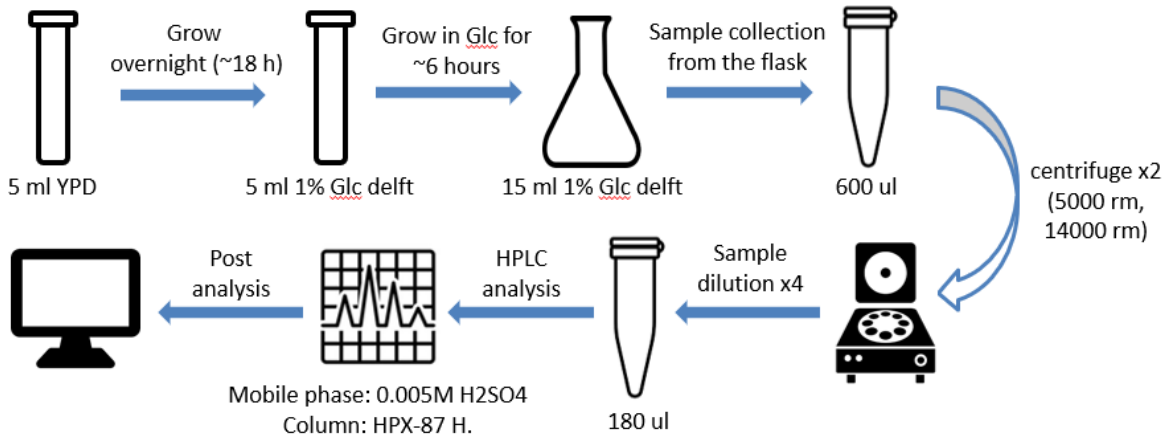


Figure 4. Data collection for physiological characterization. This scheme represents the process of sample collection and further HPLC analysis.

3.1.8.2 Instrumentation

The analysis was conducted using the Prominence-i LC-2030C Plus (Shimadzu, Japan) machine equipped with a Refractive Index Detector RID-20A (Shimadzu, Japan). The instruments were operated with LabSolutions software (Shimadzu, Japan). Rezex ROA-Organic Acid column (Phenomenex, 00H-0138-K0), protected by a guard column (Rezex 03B-0138-K0, Phenomenex) was used as a stationary phase. The mobile phase used was 5 mM sulfuric

acid. The samples were analyzed in isocratic elution mode at 45 °C with a flow rate 0.6 ml/min. Every measurement was taken in duplicates.

3.2 RESULTS

3.2.1 Identification of viable salt concentration

The goal of the plate reader experiment was to evaluate the effects of different NaCl concentrations on the growth rate of the five strains in this study. The tested NaCl concentrations included 1.4 M, 1.6 M, 1.8 M, and 2.0 M. The purpose was to identify the critical concentration for at least one of the strains and then for all of them (Table 4).

The mutant Δ*msn2* Δ*msn4* was more sensitive to the presence of NaCl in comparison with other strains. This strain was unable to grow at concentration 1.6 M NaCl or above. The strain lacking PKA catalytic subunits and Msn2p and Msn4p transcription factors deletion (Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4)* could grow until 1.8 M NaCl concentration but not above it. The Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1* strain was least sensitive to NaCl presence and was able to grow at the highest tested NaCl concentration (2.0 M) (Table 4). The strain Δ*yak* was similarly sensitive as the reference strain but grew at a faster growth rate (Figure 5).

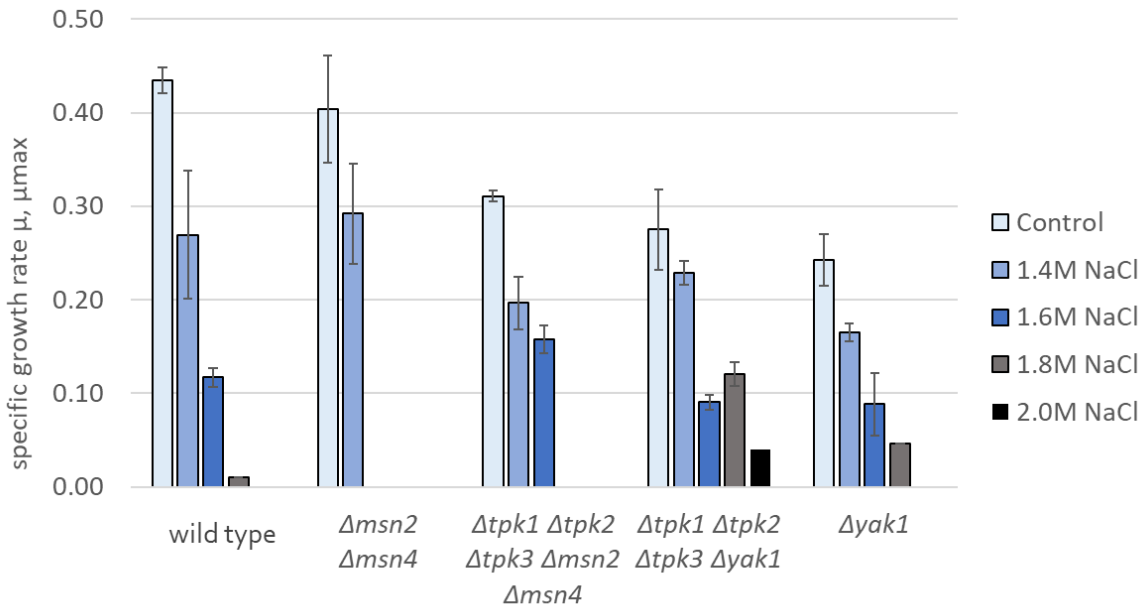


Figure 5. Comparison of specific growth rates obtained during the plate reader experiment. The experiment was performed for five strains of interest and at four NaCl concentrations (1.4 M, 1.6 M, 1.8 M, 2.0 M).

3.2.2 Strains physiological characterization at viable NaCl concentration

Although Δ*msn2* Δ*msn4* strain did not grow at 1.6 M NaCl in the plate reader experiment, we found that it was able to grow at this concentration in the shake flask. It possibly due to

large medium volume and aeration as compared with the plate reader experiment. Based on the plate reader experiment and mentioned pilot experiments in shake flask for Δ*msn2* Δ*msn4* 1.6 M NaCl was determined as a maximum viable salt concentration for physiological characterization of the strains. The physiological data (biomass, lag phase, metabolite production) was collected for all the strains that were cultivated in the presence of 1% glucose in the minimal media in the absence or presence of 1.6 M NaCl.

The reference strain growth decreased by almost 67% at 1.6 M NaCl concentration compared with the control minimal medium that lacked NaCl. The growth rate of the strain lacking Yak1p decreased by 69%, while for the strain lacking both Yak1p and PKA activity this value decreased by 40%. Under the salt stress, mutant Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4* showed less than 30% of the growth in the control condition (Table 5).

Table 4. Comparison of the specific growth rates under the control and 1.6 M NaCl stress conditions.

Strain	Condition / specific growth rate μ ($\frac{1}{h}$)		
	1% Glc	+ 1.6 M NaCl	Change, %
wild-type	0.37	0.16	*66,7%*
Δ*msn2* Δ*msn4*	0.41	0.14	*65,8%*
Δ*yak1*	0.29	0.09	*69%*
Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4*	0.37	0.11	*70,3%*
Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1*	0.25	0.15	*40%*

The lag phase is an essential step of the yeast growth process. During this phase, yeast is adapting to the environmental conditions and altering its metabolic activity in order to enter the exponential phase, where cells can rapidly grow. The reference strain's lag phase is 4-fold higher under the stress conditions than under control (Table 6). Strains, lacking Msn2p and Msn4p transcription factors, have more than a 5-time increase in the lag phase when 1.6 M NaCl is present in the environment. Although both Δ*yak1* mutants have a long lag phase

in comparison to other strains, they showed the smallest lag phase increase under the salt stress.

Table 5. Lag phase data obtained during the shake flask experiment. Five strains were tested under 1% glucose (control) and 1% Glc + 1.6 M NaCl conditions.

Strain	Lag phase (h)		
	1% glucose	+ 1.6 M NaCl	Change, fold
wild-type	2.7	17	6,3
Δ***msn2*** Δ***msn4***	4	23	5,8
Δ***yak1***	10	18	1,8
Δ***tpk1*** Δ***tpk2*** Δ***tpk3*** Δ***msn2*** Δ***msn4***	4	27	6,8
Δ***tpk1*** Δ***tpk2*** Δ***tpk3*** Δ***yak1***	8	18	2,25

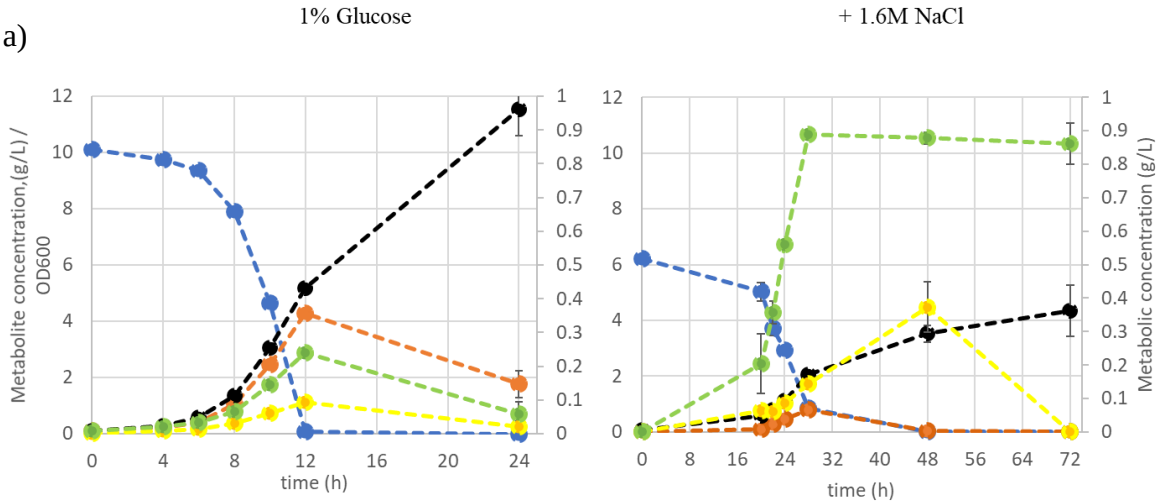
3.2.3 Yeast metabolic response of salt stress

The glucose consumption and primary yeast metabolite concentrations (ethanol, glycerol, acetic acid) were quantified using the HPLC method. The specific consumption and production rates during the exponential phase were calculated for all strains both in control and stress conditions (Table 7).

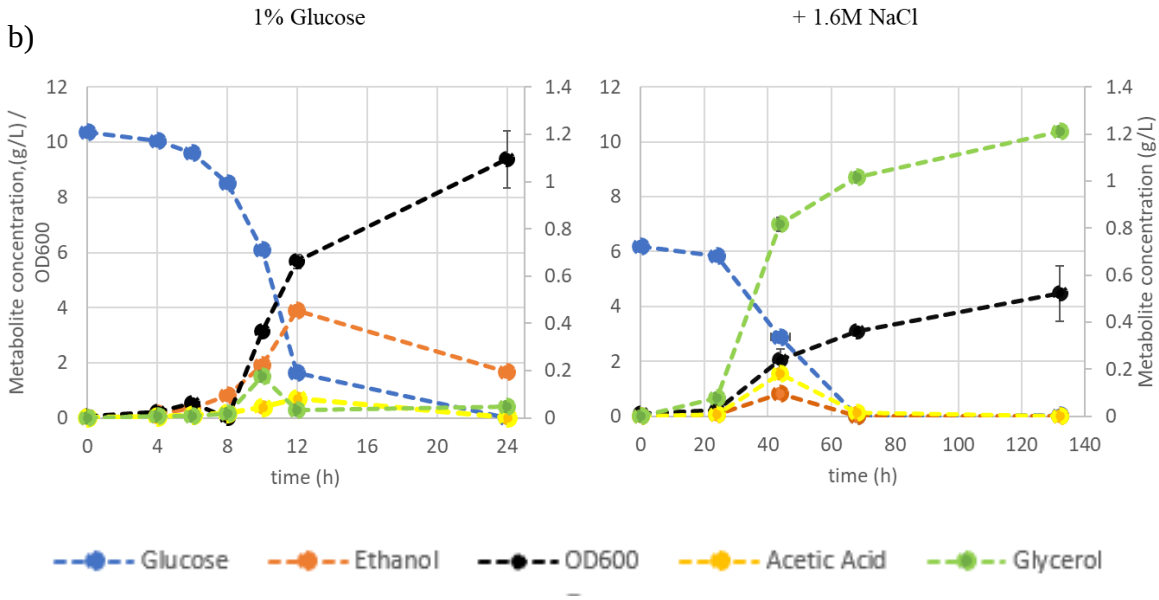
In the control condition, the reference strain consumed all the glucose in 12 hours, while it took 24 hours for other strains (Figure 6a). In total, the specific glucose consumption rate was less for the strains with deletions, especially the mutants lacking PKA activity (-35% for Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4* and -63% for Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1* in comparison to a wild-type). Consequently, the specific ethanol production rate has also decreased, especially in Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1* strain (10% of wild-type production rate). *S. cerevisiae* W303 strain's specific acetic acid production rate was 1.5-2-fold higher, than its value of other strains. Glycerol production rate decreased ~6-fold in the strains, lacking Msn2p and Msn4p and only ~1.5-fold in the strains, lacking Yak1p activity, compared to wild type.

In the presence of salt stress (1.6 M NaCl) the lag phase significantly increased, so wild-type strain glucose consumption time exceeded 24 hours (Figure 6a). For other strains, this time interval was more than 50 hours (Figure 6b,c,d,e). The glucose consumption rates decreased for all the strains. The production rate of ethanol had decreased for all strains, except *tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1*. At the same time, strains started to produce more acetic acid: we observed ~25% increase in wild-type and strains, lacking PKA activity and ~70% increase in PKA-dependent strains (Δ*yak1* and Δ*msn2* Δ*msn4*). The production rate of glycerol had increased 4-fold for the wild-type. This change was smaller for the strains, lacking Δ*yak1*: 3.5-fold for PKA-dependent strain and 2.5-fold for PKA-independent one. At the same time, Δ*msn2* Δ*msn4* and *tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4* strains had consequently the 31-fold and 7-fold increase of glycerol production rate.

a)



b)



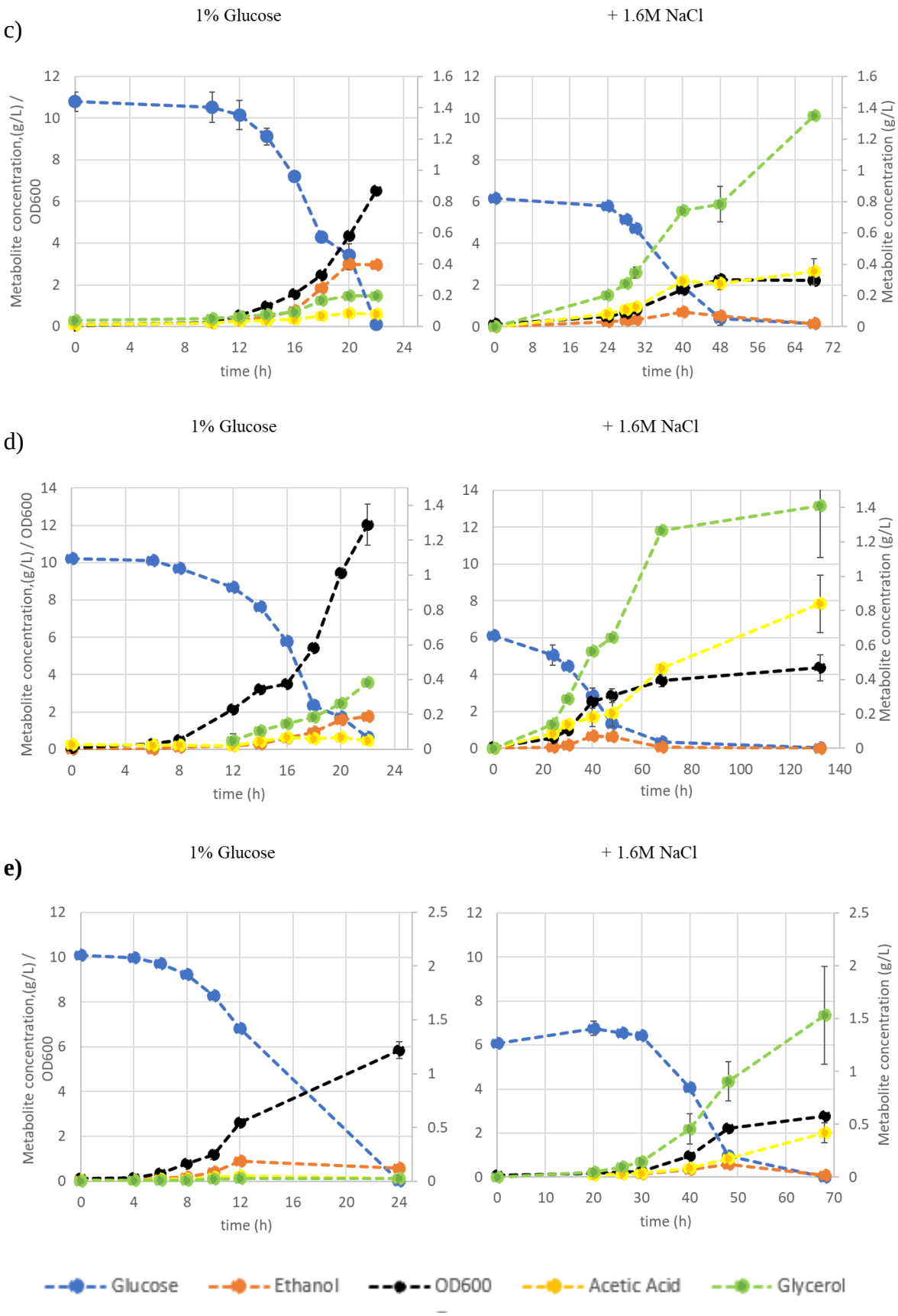


Figure 6a,b,c,d,e. Metabolic profiles of the strains under the control and the stress conditions. a) Wild-type strain b) Δ*msn2* Δ*msn4* strain c) Δ*yak1* strain d) Δ*tpk1* Δ*tpk2*

Δ*tpk3* Δ*msn2* Δ*msn4* strain e) Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1*. OD600 (black), glucose (blue), and ethanol (orange) concentration are shown on the primary axis. Acetic acid (yellow), and glycerol (green) concentration are shown on the secondary axis.

After glucose depletion, all strains had consumed ethanol, which indicates the diauxic shift. Strains Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1* and Δ*yak1* have not consumed acetic acid (Figure 6c, e). Acetic acid is a respiratory carbon source: it requires mitochondria to be consumed. All strains accumulated glycerol under salt stress.

Table 6 Specific metabolite consumption and specific production rates of the strains of interest. Samples were collected during the exponential phase of the shake flask experiment and analyzed with HPLC method.

Strain	Specific consumption rate (g/(gDCW*h))				Specific production rate (g/(gDCW*h))			
	Glucose		Ethanol		Acetic acid		Glycerol	
	Control	1.6 M NaCl	Control	1.6 M NaCl	Control	1.6 M NaCl	Control	1.6 M NaCl
wild-type	1.667	0.915	0.697	0.153	0.014	0.020	0.037	0.156
Δ*msn2* Δ*msn4*	1.379	0.499	0.615	0.126	0.008	0.029	0.004	0.125
Δ*yak1*	1.204	0.585	0.463	0.075	0.010	0.033	0.024	0.084
Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4*	1.079	0.684	0.281	0.054	0.014	0.018	0.014	0.098
Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1*	0.611	0.484	0.066	0.089	0.007	0.010	0.025	0.062

3.3 DISCUSSION

3.3.1 NaCl stress response

In this study, the NaCl stress response was investigated for five *S. cerevisiae* strains (wild-type W303 and 4 mutants). The first aim was to investigate the viability of the strains under various salt concentrations, performing the experiment in the microplate reader. During this experiment, strain Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1* proved to be the most stress tolerant (Figure 5). The mutant strain Δ*msn2* Δ*msn4* was the only strain that stopped growing at 1.6 M NaCl in the plate reader. Another vulnerable strain is Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4*, that stopped to grow at 1.8 M NaCl in plate reader experiment and showed less than 30% of initial growth in the shake flask. It happens because strains lacking Msn2p and Msn4p transcriptional factors loose viability under the salt stress, because many of the genes included in stress response are activated by these transcriptional factors (Gasch et al., 2000). So, in the presence of NaCl Δ*msn2* Δ*msn4* strains are neither stress-resistant nor capable of the same growth as in the normal conditions, as they fail to accumulate stress-regulated messages (Martinez-Pastor et al., 1996).

The major role of PKA is to eliminate the expression or function of growth-inhibitory genes, activated by Msn2p and Msn4p, including *YAK1* (Smith, 1998). Thus, in the absence of Yak1p the growth is not inhibited (Figure 2). Moreover, the activity of Msn2p and Msn4p in Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1* strain is not inhibited by PKA, so stress-response genes expression is possible (Figure 2). That explains, why this strain is a bit less stress sensitive.

For the next experiment, 1.6 M NaCl concentration was chosen as a stress condition: it is critical for the studied strains, however, they are viable (Figure 5). The effect of the salt stress and gene deletions on the growth was studied during the shake flask experiment. The results showed, that gene deletions do not significantly affect the growth under the control and salt stress conditions. At the same time, salt stress has a negative impact (40-70%) on all the strains in this study

Salt stress also affected the duration of the lag phase: it has increased from 1.8-fold to 6.8-fold, depending on the strain (Table 5). However, the lag phase difference among the studied strains was not significant. Thus, for all the strains, the growth rate and lag phase values has decreased under the salt conditions and none of the mutant strains showed significant deviation.

Since the deletion do not cause any visible effects on the growth under the control and salt stress conditions, it is possible to evaluate the difference in glucose consumption rates and metabolic production for wild type and mutant strains. The strains lacking PKA activity showed decreased glucose consumption rate: -35% for Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4* and -63% for Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1* in comparison to a wild type (Table 6). This change is less for other strains: 17% for Δ*msn2* Δ*msn4* and 28% for Δ*yak1*. The PKA is one of the major glucose-sensing kinases (Lee et al., 2008), so, most probably, the loss of its function affects the growth rate.

Strains, lacking Δ*yak1*, as well as Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4* strain, are not able to consume acetic acid both under control and stress conditions. Yak1p is required for the cell cycle arrest when the glucose is depleted in order to switch to another carbon source (diauxic shift) (Garrett et al., 1991). Probably, strains lacking Yak1p are not able to switch to acetic acid source.

All strains showed the increased accumulation of glycerol under osmotic stress (Table 6). This indicates activation of the HOG pathway – the common osmotic stress response mechanism (Hohmann, 2002). Since both wild-type and strains with deletions have increased glycerol accumulation, HOG-pathway regulation is not directly dependent on PKA, Msn2p/Msn4p or Yak1p.

SUMMARY

Deletion of *MSN2/MSN4* or *YAK1* makes the triple Δ*tpk* deletion strain viable.

PKA, *MSN2*, *MSN4* and *YAK1* gene deletions do not significantly affect the growth under the control and salt stress conditions. At the same time, salt stress has a negative impact (40-70%) on all the strains in this study.

Glucose consumption decreased in the mutant strains both under control and salt stress conditions. PKA-independent strains (Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*msn2* Δ*msn4* and Δ*tpk1* Δ*tpk2* Δ*tpk3* Δ*yak1)* have the highest decrease in glucose consumption rate indicating role of PKA in glucose metabolism in yeast.

Under osmotic stress, all tested strains showed the accumulation of glycerol: that indicates that the MAP-HOG pathway was activated. Efficient production of glycerol under salt stress likely suggests that the HOG pathway is not directly dependent on PKA or Msn2p/Msn4p or Yak1p**.**

ACKNOWLEDGEMENTS

This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 668997, and the Estonian Research Council (grant PUT1488P).

The strained used in the thesis work were kindly provided by Professor Juana M Gancedo's lab at the Instituto de Investiganciones Biomédicas "Alberto Sols' CSIC-UAM, Madrid, Spain.

REFERENCES

Bisschops, M. M. M., Zwartjens, P., Keuter, S. G. F., Pronk, J. T., & Daran-Lapujade, P. (2014). To divide or not to divide: A key role of Rim15 in calorie-restricted yeast cultures. *Biochimica et Biophysica Acta - Molecular Cell Research*. https://doi.org/10.1016/j.bbamcr.2014.01.026

Blomberg, A., & Adler, L. (1992). Physiology of osmotolerance in fungi 1. *Advances in Microbial Physiology*. https://doi.org/10.1016/s0065-2911(08)60217-9

Borodina, I., & Nielsen, J. (2014). Advances in metabolic engineering of yeast Saccharomyces cerevisiae for production of chemicals. In *Biotechnology Journal*. https://doi.org/10.1002/biot.201300445

Broach, J. R. (2012). Nutritional control of growth and development in yeast. In *Genetics*. https://doi.org/10.1534/genetics.111.135731

Calahan, D., Dunham, M., Desevo, C., & Koshland, D. E. (2011). Genetic analysis of desiccation tolerance in Saccharomyces cerevisiae. *Genetics*. https://doi.org/10.1534/genetics.111.130369

Costanzo, M., VanderSluis, B., Koch, E. N., Baryshnikova, A., Pons, C., Tan, G., Wang, W., Usaj, M., Hanchard, J., Lee, S. D., Pelechano, V., Styles, E. B., Billmann, M., Van Leeuwen, J., Van Dyk, N., Lin, Z. Y., Kuzmin, E., Nelson, J., Piotrowski, J. S., … Boone, C. (2016). A global genetic interaction network maps a wiring diagram of cellular function. *Science*. https://doi.org/10.1126/science.aaf1420

Garrett, S., & Broach, J. (1989). Loss of Ras activity in Saccharomyces cerevisiae is suppressed by disruptions of a new kinase gene, YAKI, whose product may act downstream of the cAMP-dependent protein kinase. Genes & Development. https://doi.org/10.1101/gad.3.9.1336

Garrett, S., Menold, M. M., & Broach, J. R. (1991). The Saccharomyces cerevisiae YAK1 gene encodes a protein kinase that is induced by arrest early in the cell cycle. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.11.8.4045

Goffeau, A., Barrell, G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H., & Oliver, S. G. (1996). Life with 6000 genes. *Science*. https://doi.org/10.1126/science.274.5287.546

Hartwell, L. H., Mortimer, R. K., Culotti, J., & Culotti, M. (1973). Genetic control of the cell division cycle in yeast: V. Genetic analysis of cdc mutants. *Genetics*.

Hohmann, S. (2002). Osmotic adaptation in yeast-control of the yeast osmolyte system. *International Review of Cytology*. https://doi.org/10.1016/S0074-7696(02)15008-X

http://www.pangloss.com/seidel/Protocols/ODvsCells.html OD660 vs Number of cells

Ivorra, C., Pérez-Ortín, J. E., & Del Olmo, M. lí. (1999). An inverse correlation between stress resistance and stuck fermentations in wine yeasts. A molecular study. *Biotechnology and Bioengineering*. https://doi.org/10.1002/(SICI)1097-0290(19990920)64:6<698::AID-BIT9>3.0.CO;2-Z

Kumar, R., & Lahtvee, P.J. (2020). Proteome overabundance enables respiration but limitation onsets carbon overflow. bioRxiv 2020.02.20.957662; https://doi.org/10.1101/2020.02.20.957662

Lee, P., Cho, B.-R., Joo, H.-S. and Hahn, J.-S. (2008), Yeast Yak1 kinase, a bridge be-tween PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4. Molecular Microbiology, 70: 882-895. doi:10.1111/j.1365-2958.2008.06450.x

Livas, D., Almering, M. J. H., Daran, J. M., Pronk, J. T., & Gancedo, J. M. (2011). Transcriptional responses to glucose in Saccharomyces cerevisiae strains lacking a functional protein kinase A. *BMC Genomics*. https://doi.org/10.1186/1471-2164-12-405

Martínez-Pastor, M. T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H., & Estruch, F. (1996). The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *The EMBO Journal*. https://doi.org/10.1002/j.1460-2075.1996.tb00576.x

Milo et al. Nucl. Acids Res. (2010) 38 (suppl 1): D750-D753

Serrano, R., J. A. Márquez, and G. Ríos. 1997. Crucial factors in salt tolerance., p. 147-169. *In* S. Hohmann and W. H. Mager (ed.), Yeast stress responses. R. G. Landes, Austin, Tex.

Waskiewicz, A. J., & Cooper, J. A. (1995). Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Current Opinion in Cell Biology*. https://doi.org/10.1016/0955-0674(95)80063-8

Werner-Washburne, M., Brown, D., & Braun, E. (1991). Bcy1, the regulatory subunit of cAMP-dependent protein kinase in yeast, is differentially modified in response to the physiological status of the cell. *Journal of Biological Chemistry*.

Appendix

I. Glossary

Caret The bar (or other symbol) marking the active editing point.	Sisestusmärk Märk, mis märgib teksti sisestamise asukohta.
Template A gauge, pattern, or mold, commonly a thin plate or board, used as a guide to the form of the work to be executed.	Mall Näidik, muster või valuvorm, mis esitab täitmisele võetava töö struktuuri.

NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC

I, Nadezhda Chulkova

(*author's name*)

1. herewith grant the University of Tartu a free permit (non-exclusive licence) to

reproduce, for the purpose of preservation, including for adding to the DSpace digital archives until the expiry of the term of copyright,

Characterization of *Saccharomyces cerevisiae* and its cAMP dependent protein kinase A deletions in response to saline stress.

(*title of thesis*)

supervised by Rahul Kumar, PhD, Petri-Jaan Lahtvee, PhD

(*supervisor's name*)

2. I grant the University of Tartu a permit to make the work specified in p. 1 available to the public via the web environment of the University of Tartu, including via the DSpace digital archives, under the Creative Commons licence CC BY NC ND 3.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work until the expiry of the term of copyright.

3. I am aware of the fact that the author retains the rights specified in p. 1 and 2.

4. I certify that granting the non-exclusive licence does not infringe other persons' intellectual property rights or rights arising from the personal data protection legislation.

Nadezhda Chulkova

20.05.2020