İstanbul Ecz. Fak. Derg. / J. Fac. Pharm. Istanbul
45(2) 2015 pp.165-179

Physiological and genetic analysis of cellular sodium and lithium response/resistance behavior using the yeast *Saccharomyces cerevisiae* as a model organism

Ş. Hande Tekarslan [1,2,3], Ceren Alkım [1,2], Carola Hunte [4], Z. Petek Çakar[1,2,*]

[1]Department of Molecular Biology & Genetics, Faculty of Science & Letters, Istanbul Technical University, Istanbul, Turkey
[2]Dr. Orhan Öcalgiray Molecular Biology, Biotechnology and Genetics Research Center (ITU-MOBGAM), Istanbul Technical University, Istanbul, Turkey
[3]Department of Pharmaceutical Technology, Faculty of Pharmacy, Istanbul University, Istanbul,Turkey
[4]Institute for Biochemistry and Molecular Biology, ZBMZ, BIOSS Centre for Biological Signalling Studies, University of Freiburg, Freiburg, Germany

Abstract: The yeast *Saccharomyces cerevisiae* is a simple eukaryote and an excellent model organism for molecular biology. In this study, a NaCl-resistant *S. cerevisiae* mutant obtained by inverse metabolic engineering was used as a model to investigate responses and resistance behavior to NaCl, LiCl, KCl, TMA, spermine and sorbitol stresses., at physiological and genetic levels. The physiological spot test results revealed that the NaCl-resistant yeast mutant showed higher resistance to LiCl and NaCl. Gene expression analysis by qRT-PCR revealed that *ENA6* and *NHA1* genes of the mutant were induced in the absence and presence of LiCl and NaCl. The dysfunction of Na^+/H^+ antiporters are related to several diseases such as hypertension, epilepsy, postischemic myocardial arrhythmia, gastric and kidney disease, diarrhea, and glaucoma. Thus, the NaCl-resistant yeast mutant could be used to understand cellular sodium and lithium resistance mechanisms and the function of Na^+/H^+ antiporters also in higher eukaryotic organisms, including humans.

Key words: *Saccharomyces cerevisiae*, stress resistance, salt-resistance , lithium resistance, *NHA1*

Introduction

Yeasts are one of the simplest eukaryotic organisms. Particularly the yeast *Saccharomyces cerevisiae* is an excellent model organism for eukaryotes with many conserved essential cellular processes, easy transformation,

***Correspondence:** cakarp@itu.edu.tr

fast cultivation, and efficient expression of heterologous genes (Yanagida, 2002; Müller&Grossniklaus, 2010). A better understanding of salt stress-resistance, ion transporters and their regulation would have considerable practical value. Ionic balance is regulated in humans by several mechanisms. For example, control of water flux plays an important role in regulating the concentration of Na^+ in the human cardiovascular system (Page&Di Cera, 2006). Medical studies showed that inherited defects in ion homeostasis contribute to the risk of high blood pressure, and for agricultural applications, the understanding of the proteins involved in ion homeostasis may permit genetic engineering of plants and crops for higher productivity (Mulet *et al*.,1999). Sodium chloride is very important for human biology and, K^+ and Na^+ ions are vital for the physiological function of cells. Evolutionarily adaptation to hyperosmotic stress is a highly conserved mechanism and all cell types have a strategy to adapt to the changes in the osmolarity of the surrounding medium which is fundamental to life. (Posas *et al*., 2000; Ferraris *et al*., 2001). Na^+/H^+ antiporters have an important role for the mammalian kidney to maintain the homeostasis of osmolality and the electrolyte concentrations of the circulating fluid (Lam *et al*., 2004). Homeostasis of Li^+ in yeast is maintained by multiple transport pathways that also transport Na^+. Lithium is a widely used monovalent cation to treat manic bipolar disorder. Its antimanic and antidepressant effects require days to weeks to appear, and lithium affects gene expression in the central nervous system (Manji *et al*., 1995; Masuda *et al*., 2000). The aim of this study was to investigate the cellular response and resistance mechanisms to sodium and lithium, by using *S. cerevisiae* as a model organism.

Materials and Methods

High Pure RNA Isolation Kit (Cat. No. 11 828 665 001) and LightCycler® 480 DNA SYBR Green I Master Kit (Cat. No. 04 707 516 001) were purchased from Roche. Spermine, Tetramethylammonium, lithium chloride and potassium chloride were purchased from Sigma-Aldrich. Sodium chloride was purchased from Carlo Erba. Sorbitol was purchased from Merck.

Growth of the *S. cerevisiae* Reference Strain CEN.PK 113.7D and the Salt resistant Mutant Strain 'T8';

The salt-resistant mutant strain T8 was obtained by inverse metabolic engineering methods.

Cultivation of the cells;-80ºC stock cultures of the *S. cerevisiae* reference strain CEN.PK 113.7D and the salt resistant mutant strain 'T8' were inoculated into 10 mLYPD mediumin 50-mL culture tubes. After overnight incubation, their OD_{600} values were adjusted to 0.25 and inoculated into 100 mL Yeast Minimal Medium (YMM) at 30°C at a shaking speed of 150 rpm.

Growth Analysis; CEN.PK 113.7D and the salt resistant mutant strain 'T8' were grown in 100 mL YMM without and with 0.5M, 0.7 M, 0.9M NaCl in 500 mL flasks starting with OD_{600} value of 0.25at 30 ºC, 150 rpm. The maximum specific growth rate (μ_{max}) corresponds to to the maximum slope from this semi-logarithmic representation and was calculated from the logarithm of the OD_{600} values.

Spot test of CEN.PK 113.7D; Spot test was used to determine the resistance or sensitivity of yeast strains to Tetramethylammonium (TMA), Spermine, NaCl, KCl and LiCl, Sorbitol, and pH effect. Cells were cultivated in 50 mL-culture tubes containing 10-mL liquid YMM at 30ºC and 150 rpm until OD_{600} of 1.2. Equal numbers of yeast cells were centrifuged at 10000 rpm for 5 min and the supernatant was discarded. After the pellets were resuspended in 100 µL water, they were diluted serially as 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} in 96-well plates. Three µL from these diluted suspensions were dropped onto solid control plates and onto plates containing stress factors in their media. For pH effect, growth was tested at pH 3.6, 5.5, 6.6 and 7.9. HCl was added to the media for pH 3.6, and NaOH was used for pH 5.5, 6.6 and 7.9 (Banuelos *et al.*, 1998).

Real-Time qRT-PCR

Total RNA isolation from yeast cells (CEN.PK 113.7D); Total RNA was isolated from untreated cells and cells treated with 0.7 M NaCl and 5 mM LiCl with High Pure RNA isolation kit. The reference strain and T8 were grown in 10 mL YMM at 30 ˚C, 150 rpm until they reached OD_{600} of 1. Cells

were centrifuged at 13000 rpm for 5 min and supernatants were discarded. Purification was carried out following manufacturer's instructions. Total RNA concentrations were measured by Nanodrop.

*cDNA synthesis;*cDNA was synthesized from total RNA samples by Transcriptor High Fidelity cDNA Synthesis Kit (Cat. No. 05 081 955 001) following manufacturer's instructions.

Primer design of genes; The primer design of genes were analyzed by "Primer3 Plus Software" and "Amplify3 - 3.1.4 version - MacOS X" software.

Real-time qRT-PCR applications; *ENA6* and *NHA1* gene expression levelswere quantified by Real-Time qRT-PCR in the absence and presence of lithium and sodium by using Roche-LightCycler® 480 DNA SYBR Green I Master Kit (Cat. No. 04 707 516 001). qRT-PCR experiments were performed as 20 µL reactions under following cycling conditions: denaturation for 10 sec at 95°C, 45 cycles of 10 sec at 95°C, 15 sec at 56°C, 20 sec at 72°C, one cycle of melting for 5 sec at 95°C, 1 min at 65°C and denaturation step at 40°C for 10 sec in Light Cycler 480 Equipment (Roche).The internal control housekeeping gene was chosen as beta-actin (*ACT1*). The relative expression levels of *ENA6* and *NHA1* were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The efficiency, error and slope values of each standard curve and the melting curves of each qRT-PCR were analyzed in detail (Dorak, 2006).

Results

Growth physiology of CEN.PK 113.7D and T8 in the presence of 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl; Growth curves of CEN.PK 113.7D and T8 in the absence and presence of 0.5 M NaCl, 0.7 M NaCl, 0.9 M NaCl were obtained by measuring OD_{600} values every 1.5 h (Tables 1&2).

Table 1: Average OD_{600} values of the reference strain (Wt) CEN.PK 113.7D in the absence and presence of 0.5 M NaCl, 0.7 M NaCl, 0.9 M NaCl.St Dev indicates standard deviations.

OD_{600}	Wt	St Dev	Wt+0.5 M NaCl	St Dev	Wt+0.7 M NaCl	StDev	Wt+0.9 M NaCl	St Dev
t=0 h	0.35	0.01	0.35	0.01	0.09	0.00	0.01	0.00
t=1 h	0.58	0.01	0.47	0.01	0.44	0.01	0.45	0.02
t=2 h	1.21	0.03	0.61	0.05	0.57	0.02	0.52	0.00
t=3 h	2.52	0.20	0.79	0.01	0.60	0.05	0.68	0.00
t=4 h	3.58	0.18	0.83	0.01	0.79	0.00	0.71	0.06
t=5 h	4.16	0.24	0.91	0.00	0.97	0.07	0.80	0.00
t=6 h	5.73	0.21	1.10	0.07	1.14	0.03	0.93	0.00

Table 2: Average OD_{600} values of T8in the absence and presence of 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl. St Dev indicates standard deviations.

OD_{600}	T8	St Dev	T8+0.5 M NaCl	St Dev	T8+0.7 M NaCl	St Dev	T8+0.9 M NaCl	St Dev
t=0 h	0.35	0.02	0.32	0.02	0.29	0.00	0.32	0.02
t=1 h	0.52	0.03	0.42	0.01	0.47	0.02	0.37	0.00
t=2 h	0.92	0.03	0.58	0.01	0.60	0.02	0.47	0.00
t=3 h	1.64	0.01	0.89	0.05	0.83	0.00	0.66	0.01
t=4 h	2.56	0.03	1.23	0.02	0.79	0.04	1.05	0.11
t=5 h	3.34	0.06	1.73	0.10	1.40	0.16	1.06	0.01
t=6 h	5.00	0.03	2.20	0.01	1.89	0.06	1.43	0.01

According to average OD_{600} values,maximum specific growth rates μ_{max} (h^{-1}) of the reference strain (Wt) and "T8" in YMM were calculated by growing these strains in 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl (Table 3).

Table 3: Maximum specific growth rates μ_{max} (h^{-1}) of CEN.PK 113.7D (Wt) and T8 in the absence and presence of 0.5 M NaCl, 0.7 M NaCl, 0.9 M NaCl.

	Wt	Wt+0.5 M NaCl	Wt+0.7 M NaCl	Wt+0.9 M NaCl	T8	T8+0.5 M NaCl	T8+0.7 M NaCl	T8+0.9 M NaCl
μ	0.44	0.18	0.11	0.09	0.35	0.24	0.19	0.17

In evolutionary engineering studies, improved phenotype or property often indicates increased fitness. Maximum growth rate (μ_{max}) of microbial cells for toxic concentrations of certain chemical compounds are used as a fitness criteria for biotechnology (Dragosits& Mattanovich, 2013). μ_{max} values of T8 were significantly higher than that of the reference strain (Wt) (Table 3). Growth inhibition was clearly observed in the reference strain in the presence of 0.9 M NaCl. T8 (μ=0.17) obviously grew better than the reference strain at 0.9 M NaCl stress.

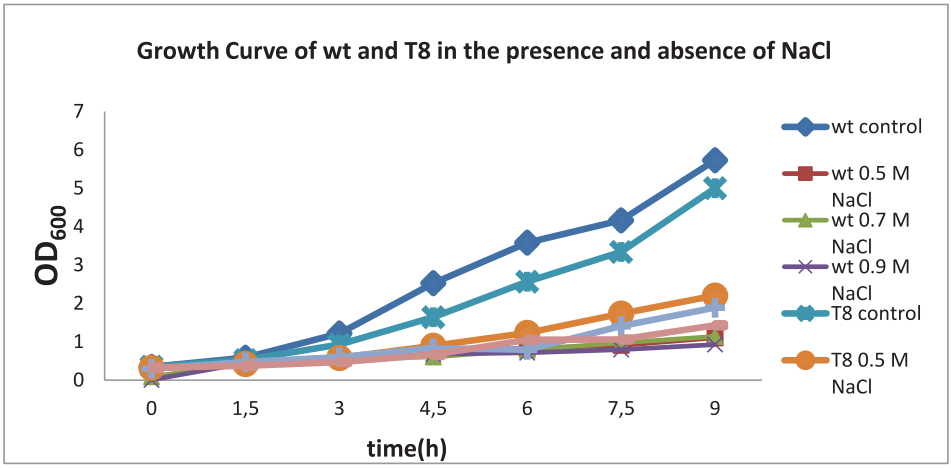

Figure 1: Growth curves of the reference strain (Wt) and T8 in the absence and presence of 0.5 M, 0.7 M,0.9 M NaCl

It can be concluded that T8 is resistant to 0.5 M NaCl, 0.7 M NaCl and 0.9 M NaCl (Figure 1).

Investigation of membrane potential-related properties using tetramethylammonium (TMA) and spermine

The reference strain and T8 were tested for their potential resistance against cationic drugs, Tetramethylammonium (TMA) (0.2, 0.3, 0.4, 0.5 and 0.6 M) and spermine (0.3, 0.4, 0.5, 0.6 and 0.7 mM). Salt resistant strain (T8) showed slight resistance to 0.3 mM and 0.4 mM spermine, however,no resistance was observed against TMA (Figures 2&3).

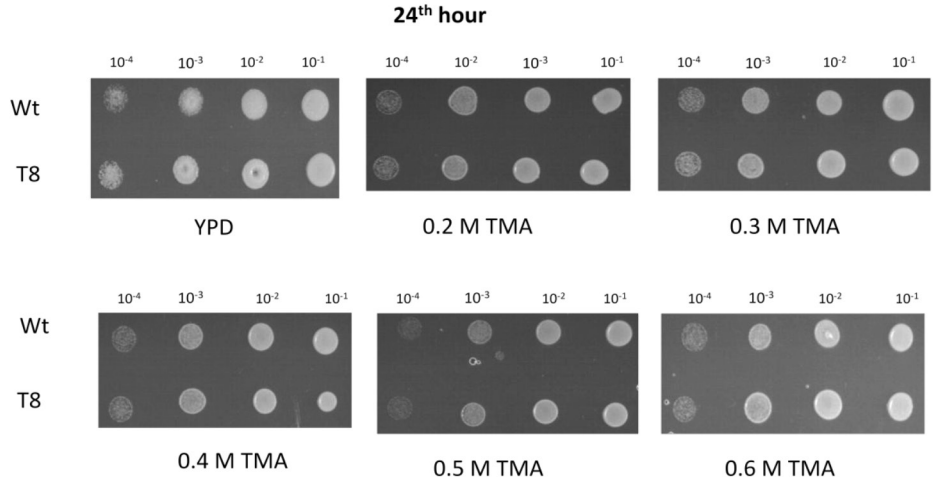

Figure 2: Spot test results of the reference strain (Wt) and T8 in YPD containing different concentrations of TMA (24th hour).

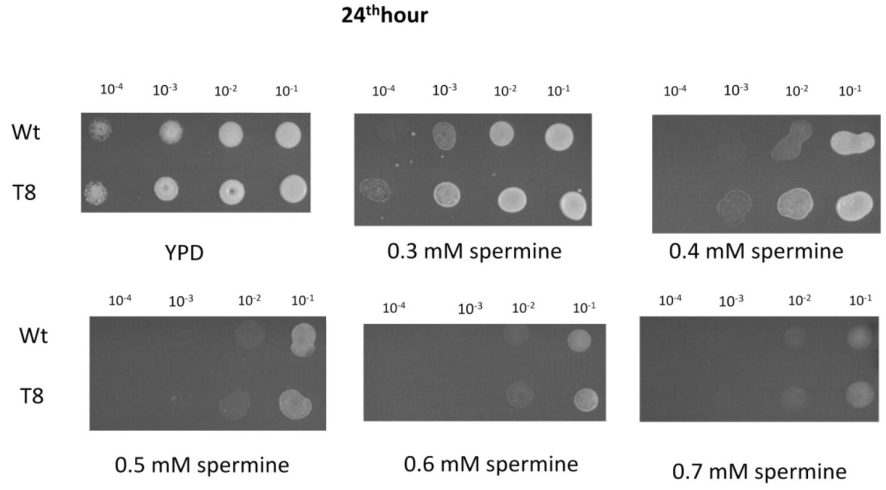

Figure 3: Spot test results of the reference strain (Wt) and T8 in YPD containing different concentrations of spermine (24th h).

If there was an alteration in the electrochemical gradient, mutants would be abnormally sensitive to toxic cations. Altered tolerance to diverse toxic cations, differing in the specific uptake transport system

and toxicity mechanisms, would reflect a change in the electrochemical gradient (Barreto *et al.*, 2011). Madrid *et al*., (1998) and Mulet *et al*., (1999) suggested that yeast mutants were sensitive to cationic drugs when K^+ uptake is impaired. Kinclova-Zimmermannova *et al*. (2006) concluded that cationic drugs could be the marker for membrane potential change. Also Munson *et al*., (2004) showed that mutants which have amutation in their Na^+ pathway hadincreased sensitivity to cationic compounds such as hygromycin B or tetramethylammonium and this could be due to the increased electrochemical potential at the plasma membrane. As the NaCl-resistant mutant T8 is not sensitive to TMA and spermine, T8 does not seem to have an altered membrane potential.

Resistance of CEN.PK 113.7D and T8 to NaCl, KCl, LiCl and Sorbitol

The salt-resistant mutant T8 showed resistance to 0.7 M, 1 M and 1.3 M NaCl and also high resistance to LiCl. Although the reference strain was sensitive to even 5 mM LiCl, T8 was resistant to 5, 10 and 15 mM LiCl (Figure 4).

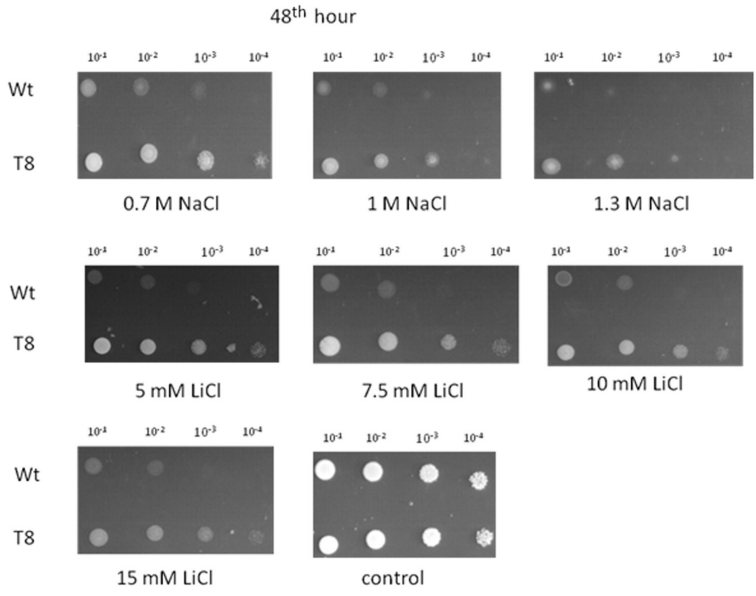

Figure 4: Spot test results of the reference strain(Wt) and T8 in YMM containing different concentrations of NaCl and LiCl (48th hour).

Concentrations of 50 mM, 100 mM, 250 mM, 500 mM and 1 M KCl did not affect the growth of the reference strain and T8. These concentrations of KCl did not cause any growth difference for the reference strain and T8 (Figure 5).

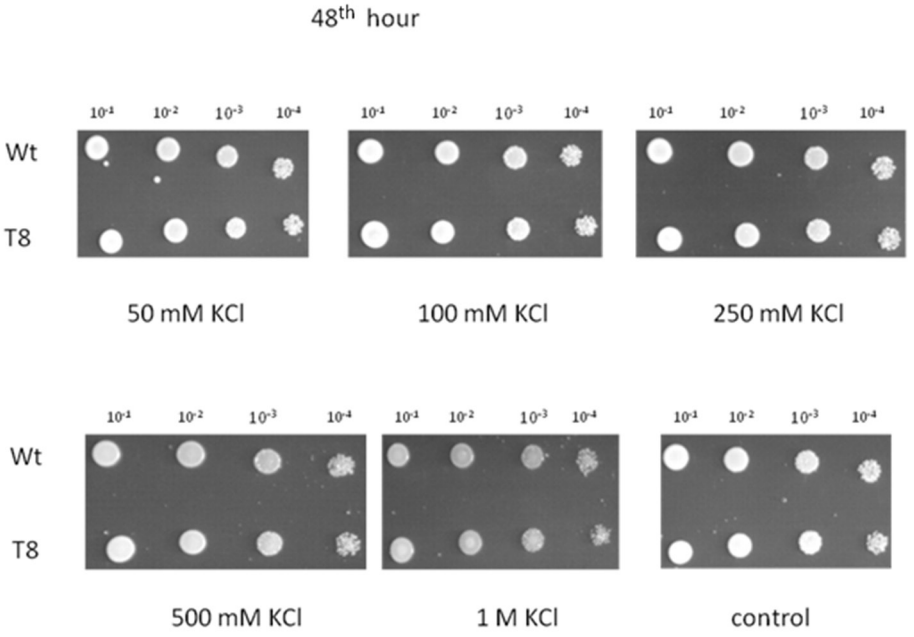

Figure 5: Spot test results of the reference strain (Wt) and T8 in YMM containing different concentrations of KCl (48th hour).

The reference strain and T8 were tested for their possible resistances to 1.25, 1.5 and 1.75 M KCl and %25, %30 and %35 w/v sorbitol.

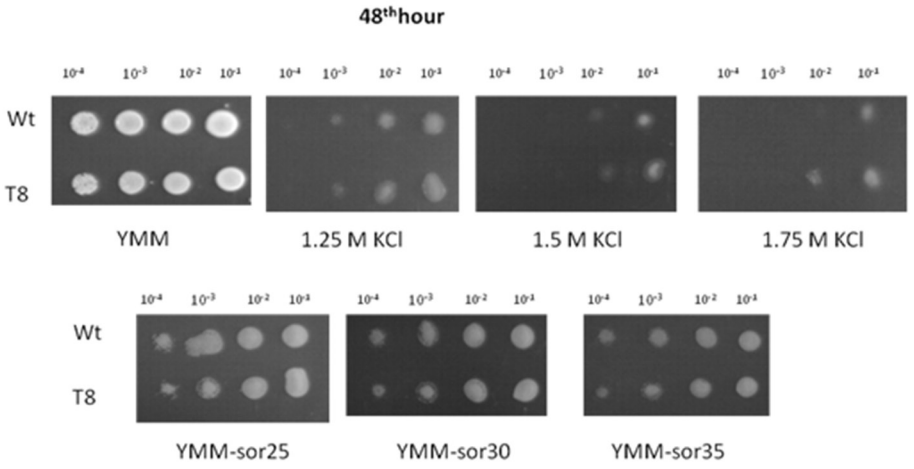

Figure 6: Spot test results of the reference strain and T8 in YMM containing different concentrations of KCl (48th hour).

At higher concentrations of KCl, both the reference strain's and T8's growth were diminished by KCl stress. Salt resistant mutant grew better than the the reference strain at 1.75 M KCl (Figure 6). The lithium sensitivity of yeast cells reflects the toxicity of the cation, while high potassium and sorbitol sensitivities often mainly reflectthe osmotic effect (Figures 4&5&6). NaCl sensitivity reflects both osmotic and cationic stress. The sodium cation is more toxic than potassium, but far less than lithium (Maresova and Sychrova, 2010). NaCl resistant mutant "T8" was resistant to sodium and lithium stress, while potassium and sorbitol did not affect the mutant. This demonstrates that T8 has resistance to cation toxicity, but not to osmotic stress.

pH and 0.7 M NaCl stress effects

The growth capacity of yeast strains in the presence of 0.7M NaCl at different pH values were tested. T8 was highly more resistant than the reference strainat pH 3.6 with 0.7 M NaCl stress. T8 was more reistant than the reference strain at pH 5.5 and 6.6 with 0.7 M NaCl stress.There was no difference between T8 and the reference strain at pH 7.9. It can be concluded that T8 became more resistant to salt stress at acidic pH (Figure 7).

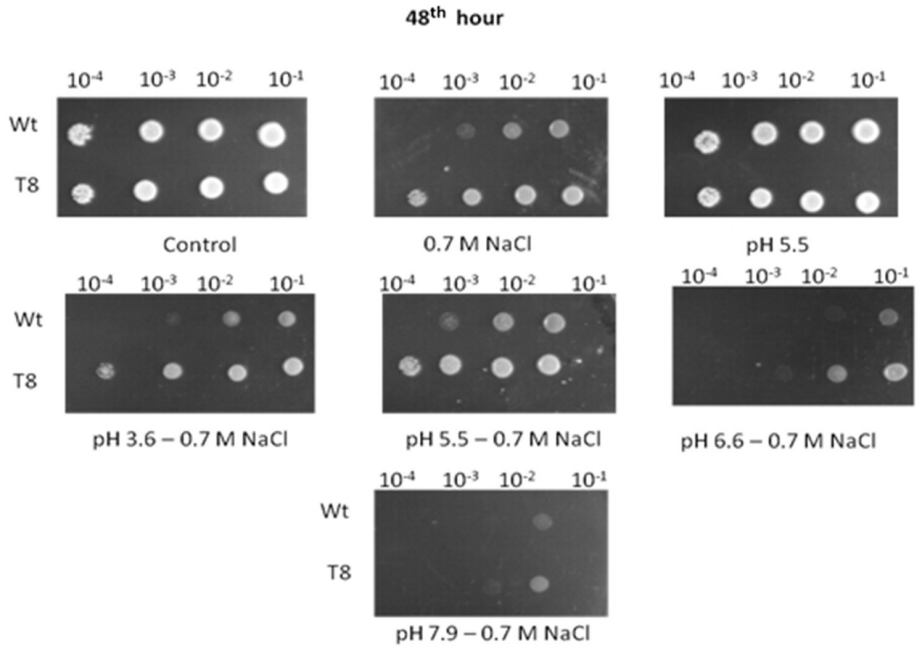

Figure 7: Spot test results of the reference strain (Wt) and T8 containing 0.7 M NaCl and at different pH range (48th hour).

Yeast has two transport systems : a cluster of up to five P-ATPases *PMR2A*/*ENA1-4* and a Na^+/H^+ antiporter, encoded by *NHA1* which pump Na^+ out of the cell. *ENA1-4* and *NHA1* are differently regulated. Osmotic stress, high extracellular pH and starvation induce *ENA6* expression. Acidic pH and neutral pH induce *NHA1* gene and induced *NHA1* increases sodium and lithium tolerance (Masuda *et al*., 2000).

Expression Level Determination of *ENA6* and *NHA1* genesfor the reference strain and T8 using qRT-PCR

ENA6 and *NHA1* gene expression levels for the reference strain and T8 were determined in the absence and presence of 5 mM LiCl and 0.7 M NaCl, using qRT-PCR.

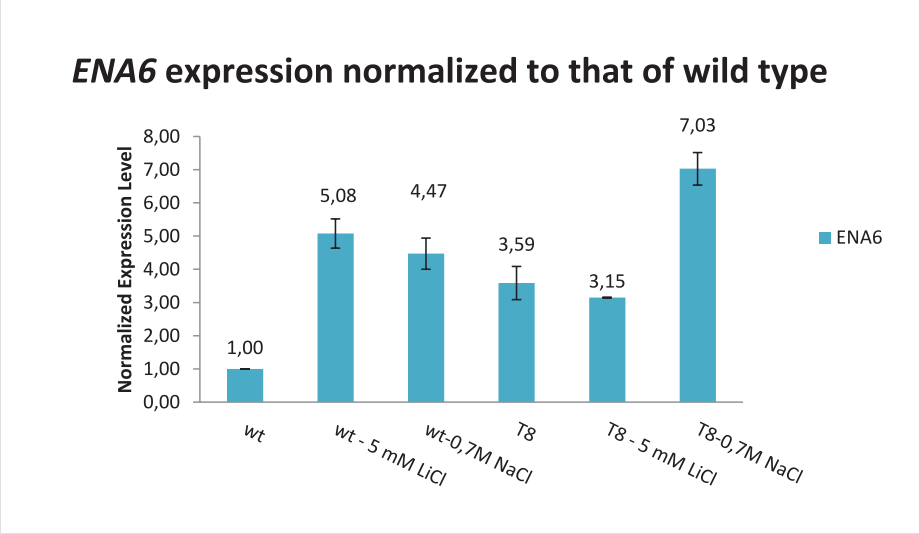

Figure 8: Expression level of *ENA6* gene in control, 0.7 M NaCl and 5 mM LiCl stress conditions.

ENA6 gene of the reference strain and T8 was upregulated in all conditions. While NaCl stress induced *ENA6* expresssion of T8 more, LiCl stress was more effective on the reference strain's *ENA6* expression (Figure 8).

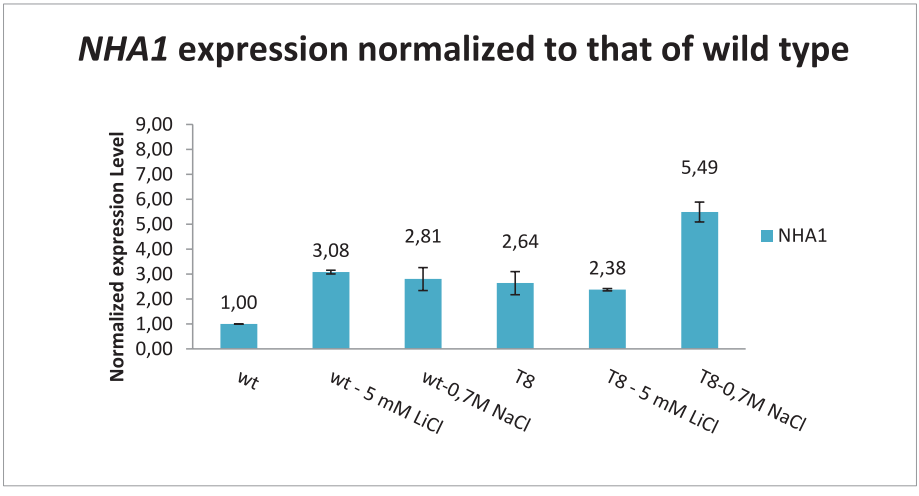

Figure 9: Expression level of *NHA1* gene in control, 0.7 M NaCl and 5 mM LiCl stress conditions.

NHA1 was induced in T8 without stress. Its expression level was highest in T8 when NaCl stress was applied. It was obvious that *ENA6* was induced more under each condition tested. Expression of *NHA1* was upregulated at Na^+ and Li^+ concentrations. Expression of *ENA6* is inducible by Na^+, Li^+ or high pH values, whereas expression of *NHA1*does not seem to be inducible by salts, pH changes or osmotic shocks (Sychrova, 20004). Banuelos *et al*. (1998) suggested that the *NHA1* transcription is lower than that of *ENA6*. In our results, both *NHA1* and *ENA6* are induced in T8, and also LiCl and NaCl stress triggered *NHA1* and *ENA6* expression. Expression level of *NHA1* was highest in T8 in the presence of NaCl stress. It was obvious that expression level of *ENA6* was higher in every condition.

Conclusion

There is anassociation between Na^+/H^+ hydrogen exchangers (NHE) dysfunction and hypertension, epilepsy, postischemic myocardial arrhythmia, gastric and kidney disease, diarrhea, and glaucoma in mammals (Brett *et al*., 2005; Hunte *et al*., 2005). *NHA1* is the yeast homologue of human Na^+/H^+ hydrogen exchangers (NHE) and *S. cerevisiae* is an appropriate model organism to study NHE.There are nearly 1000 yeast genes which are members of orthologous gene families associated with human disease (Heinicke *et al*., 2007). The salt-resistant yeast mutant could help clarify sodium and lithium pathways and *NHA1* function.

Acknowledgements

This work was supported by Istanbul Technical University Research Funds (ITU-BAP graduate student project no: 37730 to Z.P.Ç. and Ş.H.T), European Cooperation in Science and Technology Action (COST) CM0902; the Scientific and Technological Research Council of Turkey (TUBITAK project no:109T638 to Z.P.Ç); SFB 746 (German Research Foundation to C.H.); Excellence Initiative of the German Federal and State Governments (EXC 294 to C.H.); European Community's Seventh Framework Programme HEALTHF4-2007-201924, and EDICT Consortium (to C.H.). Ş. Hande Tekarslan was supported by The Scientific and Technological Research Council of Turkey -National Scholarship Programme for PhD Students.

References

Banuelos MA, Sychrova H, Bleykasten-Grosshans C, Souciet JL, Potier S (1998) The Nha1 antiporter of *Saccharomyces cerevisiae* mediates sodium and potassium efflux, *Microbiology* , **144** (10): 2749-58.

Barreto L, Canadell D, Petrezselyova S, Navarrete C, Maresova L, Perez-Valle J, Herrera R, Olier I, Giraldo J, Sychrova H, Yenush L, Ramos J, Arino J (2011) A genomewide screen for tolerance to cationic drugs reveals genes important for potassium homeostasis in *Saccharomyces cerevisiae*, *Eukaryot Cell,* **10**(9):1241-50.

Brett CL , Donowitz M, Rao R (2005) Evolutionary origins of eukaryotic sodium/proton exchangers. *American Journal of Physiology - Cell Physiology*, doi: 10.1152/ajpcell.00360.2004.

Dorak TM (2006) Real-Time PCR. 1st ed, Advanced Methods, USA.

Dragosits M, Mattanovich D (2013) Adaptive laboratory evolution – principles and applications for biotechnology. *Microbial Cell Factories,* doi: 10.1186/1475-2859-12-64.

Ferraris JD, Garcia-Perez A (2001) Osmotically Responsive Genes: The Mammalian Osmotic Response Element (ORE), *American Zoologist.*, **41**:734–742.

Heinicke S, Livstone MS, Lu C, Oughtred R, Kang F, Angiuoli SV, White O, Botstein D, Dolinski K (2007) The Princeton Protein Orthology Database (P-POD): A Comparative Genomics Analysis Tool for Biologists, *PLoS ONE,* doi: 10.1371/journal.pone.0000766.

Hunte C, Screpanti E, Venturi M, Rimon A, Padan E, Michel H (2005) Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH, *Nature*, **435** (7046):1197-202.

Kinclova-Zimmermannova O, Gaskova D, Sychrova H (2006) The Na^+, K^+/H^+-antiporter Nha1 influences the plasma membrane potential of *Saccharomyces cerevisiae*, *FEMS Yeast Research*, **6**: 792-800.

Lam AK, Ko BC, Tam S, Morris R, Yang JY, Chung SK, Chung SS (2004) Osmotic response element-binding protein (OREBP) is an essential regulator of the urine concentrating mechanism, *The Journal of Biological Chemistry*, 279 (46):48048-54.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods* , **25**(4):402-8.

Madrid J, Hernández F, Pulgar MA, Cid JM (1998) Effects of citrus by-product supplementation on the intake and digestibility of urea sodium hydroxide-treated barley straw in goats, *Small Ruminant Reearch.*, **28**: 241-248.

Manji, HK, Lenox RH (1998) Lithium: A Molecular Transducer of Mood-Stabilization in the Treatment of Bipolar Disorder, *Neuropsychopharmacology*, **19**, 161–166.

Maresova L, Sychrova H (2010) Genetic interactions between the Arl1 GTPase and

intracellular Na^+/H^+ antiporters in pH homeostasis and cation detoxification, *FEMS Yeast Research,* **10**, 802-811.

Masuda CA, Ramirez J, Pena A, Montero-Lomelí M (2000) Regulation of monovalent ion homeostasis and pH by the Ser- Thr protein phosphatase SIT4 in *Saccharomyces cerevisiae*, *The Journal of Biological Chemistry*, **275**: 30957–30961.

Mulet JM, Alejandro S, Romero C, Serrano R (2004) The trehalose pathway and intracellular glucose phosphates as modulators of potassium transport and general cation homeostasis in yeast, *Yeast ,* **21**: 569–582.

Munson AM, Love SL, Shu J, Palanivel VR, Rosenwald AG (2004) ARL1 participates with ATC1/LIC4 to regulate responses of yeast cells to ions. *Biochemical and Biophysical Research Communications,* **315**(3):617-23.

Müller B, Grossniklaus U (2010) Model organisms — A historical perspective, *Journal of Proteomics,* **73**:2054-2063.

Page MJ, Di Cera E (2006) Role of $Na^+$and K^+ in Enzyme Function. *Physiological Reviews,* **86**: 1049–1092.

Posas F, Chambers JR, Heyman JA, Hoeffler JP, de Nadal E, Arino J (2000) The transcriptional response of yeast to saline stress, *The Journal of Biological Chemistry,* **275**: 17249–17255.

Sychrova H (2004) Yeast as a model organism to study transport and homeostasis of alkali metal cations, *Physiological Research,* **53**,1:S91-8.

Yanagida M (2002). The model unicellular eukaryote, *Schizosaccharomyces pombe*. *Genome Biology*, doi:10.1186/gb-2002-3-3-comment2003.