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## Salt stress resistance in yeast is heterogeneous.

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### Abstract

*Individual cells within genetically-pure microbial cultures exhibit marked differences in their stress resistances. Such non-genetic variation in stress resistance could be critical for the fitness and survival of species in the wild or laboratory settings, but its molecular basis is poorly understood. Here we used a suite of novel assays to provide an explanation for heterogeneous salt resistance in yeast culture. Specific mutants from a complete yeast deletion strains collection were tested for altered heterogeneity and we identified a gene product that contribute to heterogeneous resistance to salt stress (GPD 1p) and ENA 1 that may be in a gene network that buffers heterogeneity in the wild *Saccharomyces cerevisiae*. Cell cycle progression was found to be principal parameter underpinning differential salt resistance and cell-cycle-dependent NaCl resistance is largely removed in the absence of GPD 1. Also, we analyzed the heterogeneous resistance to hyperosmotic shock that proved not to be a binary phenomenon (dead/alive) and that produces non-cultivable cells.*

Keywords: phenotypic heterogeneity in long- and short-term salt stress, heterogeneity ratio, cell cycle progression, flow cytometry

### Introduction

Yeast cells are an excellent eukaryotic model for the study of the cellular mechanisms underlying heterogeneous response to salt stress, particularly because it has been shown that fungi and higher plants share the same adaptation mechanisms and yeast models may be extended to plants. Additionally, halotolerance genes are preserved in plants and yeast cells support functional expression of plant genes encoding transport systems [9, 11, 13, 14].

Phenotypic heterogeneity or non-genetic cell-to-cell variability describes variation that exists between individual cells within clonal populations and it has been proposed that such heterogeneity in collaboration with genetic diversity promote the fitness of natural fungal population [2, 16]. Phenotypic diversity in *Saccharomyces cerevisiae* is connected with various cellular parameters or processes like cell cycle progression, cell ageing, mitochondrial activity, ultradian rhythms and potentially also other epigenetic factors and stochastic variation. A variety of novel methods have been described to study heterogeneity in yeast and other organisms, a lot of them used flow cytometry and cell sorting and various forms of microscopy, but patch-clamp technique or RNA expression profiling of individual cells have also been used [1, 12].

In the last years, using flow cytometry and other experimental strategies it has been proved that heterogeneous copper resistance in yeast is not stochastic and that *SOD1* and *CUP1* genes are required to establish the cell cycle- and age-dependency of heterogeneous Cu

resistance [17]. The same group identified another two genes *VMA1* and *VMA3* that act to suppress heterogeneity in Ni resistance, and their buffering of hidden phenotypic variation can provide a novel model of ‘evolutionary capacitance’ for further studies (as it has been proposed for Hsp 90) [3, 18].

## Materials and Methods

### *Yeasts strains and salt stress conditions*

The *Saccharomyces cerevisiae* BY 4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and the isogenic mutant strains: YPR005C (*hal1Δ*), YOL064C (*hal2Δ*), YKR072C (*hal3Δ*), YJL129C (*trk1Δ*), YKR050W (*trk2Δ*), YMR126C (*tps1Δ*), YDR074W (*tps2Δ*), YOL059W (*gpd2Δ*), YDL022W (*gpd1Δ*), YML106W (*ura5Δ*), YJL165C (*hal5Δ*), YIL053W (*gpp1Δ*), YER062C (*gpp2Δ*), YJR104C (*sod1Δ*), YLL043W (*fps1Δ*), YLR113W (*hog1Δ*) were obtained from Euroscarf (Frankfurt, Germany). *S. cerevisiae* W 303-1A (*MATa ura3-52 trp1Δ2 leu2-3\_112 his3\_11 ade2\_1 can1-100*) and isogenic strain G1.9 (*ena1Δ*) were kindly provided by Alonso Rodríguez-Navarro (Escuela Técnica Superior de Ingenieros Agrónomos, Madrid, Spain). The strains were routinely maintained on YEPD agar medium.

For salt stress experiments on agar cells were grown on an orbital shaker (120 rev/min) in YEPD broth until the exponential phase ( $OD_{600} \sim 2.0$ ). The overnight cultures were diluted in and plated on salt-containing rich media plates (0 – 12% [w/v] NaCl). Plates were incubated at 30°C for 10 days and survival of cells was assayed in term of colony-forming units (CFU).

For cell-cycle-arrest experiments the overnight cultures ( $OD_{600} \sim 2.0$ ) were incubated for 120 minutes with nocodazole (Sigma, 15 µg/ml). For release from arrest, cells were centrifuged and the pellet washed twice with YEPD and resuspended in the same medium. Release from arrest was monitored by determination of budding index (BI – number of budded cells/number of total cells x 100) and only the cultures where BI > 90 were used for experiments. The colony-forming capacity of the cultures was determined on salt series YEPD plates, containing the indicated amounts of NaCl.

Osmotic shock was applied growing cells in YEPD until the exponential phase and then subjected to a saline shock (0 – 30% [w/v] NaCl) for 10 minutes at 120 rpm, 30°C. The cell viability was determined as CFU on agar plates, also by fluorescence microscopy (after propidium iodide (PI) staining; at least 300 cells were counted in each sample) and also by flow cytometric analysis of PI-stained cells.

Heterogeneity ratio (HR) is defined as the ratio of the log increases in stressor concentrations required to give one-log decreases in viability, for mutant cultures relative to wild-type culture [17]. The HR values were calculated using R software (version 1.9.0) (<http://www.sourcekeg.co.uk/cran>) as described previously.

### *Flow cytometry*

A Beckman-Coulter Altra instrument equipped with a 488-nm laser was used for analysis of propidium iodide fluorescence (PI). Flow cytometry data were analysed using WINMDI (version 2.8) software (<http://facs.scripps.edu/software.html>).

After osmotic shock cells were harvested for 2 minutes at 1200 rpm, re-suspended in phosphate-buffered saline (PBS) and stained with PI (25 µg/ml) for 15 minutes. Typically 5 x 10<sup>4</sup> cells were analyzed per sample for viability determinations. The resulting signals were processed to gather information about the sizes of the cells (forward light scatter FS Lin and side light scatter PMT1 Lin) and the intensity of fluorescence measured via a 610-nm bandpass filter (PMT4 for propidium iodide).

### *Reproducibility of the data*

Experiments were generally performed at least in duplicate or triplicate and the results from representative experiments are shown. There was some day-to-day variation in the resistance of cultures to salt (even between wild strains) but the degree of heterogeneity remained consistent from experiment to experiment. The viability after PI stain differed from experiment to experiment by no more than 10%.

## **Results and Discussion**

### *Phenotypic diversity in salt stress adaptation*

In the yeast's natural environment, the water activity can range widely and sometime rapidly, due to both external influences and the activity of yeast itself. These drastic changes in water availability document that the yeast cells should possess all the mechanisms that an eukaryotic cell requires to respond and to adapt to changes in the osmolarity of the environment [7]. The ability to survive to water stress must be an intrinsic property of the cell, which means that the appropriate survival systems are in place under these conditions, especially the mechanisms that are not costly from a bioenergetic point of view (e.g. glycerol production). In this respect it can be expected that both genetic and phenotypic diversity to provide an important insurance mechanisms for survival and adaptation of cells to high osmolarity.

However, salt causes both hyperosmotic stress as well as effects due to specific cation toxicity (chloride toxicity has not been observed in yeasts). In the response of *Saccharomyces* sp. to saline stress, different stages can be distinguished: (i) an immediate rescue mechanisms that prevent cell death after sudden change in osmolarity, (ii) primary defense processes elicited in order to set protection, repair and recovery after osmotic effect and Na toxicity and (iii) an adaptive response that allow the cell to resume growth [7]. Therefore, in yeast long-term defence responses to salt stress are based on osmotic adjustment by osmolyte synthesis (different polyols, especially glycerol) and cation transport system for sodium exclusion.

To test if cell-to-cell heterogeneity in salt stress defence response is dependent by the activity of any specific genes we screened a number of 18 mutants deficient in genes known to be involved in osmolyte production and ions homeostasis. An important premise for this work was that if differential expression of a gene product contributes to heterogeneity, then disruption of that gene should give diminished heterogeneity compared to the wild type [17]. For this purpose, dose-response curves were determined for each mutant and wild type (cultures were obtained from parallel experiments performed at the same time) and the gradients of curves for mutant versus wild-type were compared using the heterogeneity ratio (HR). A HR value lower than 1 would indicate that the deleted gene normally acts to increase heterogeneity, whereas higher heterogeneity ratio that the effect of gene is to decrease heterogeneity.

Briefly, the halotolerance genes screened can be classified in four different categories: (i) ion transporters and regulators of ion transportes (*TRK1*, *TRK2*, *ENA 1*, *HAL1*, *HAL3*, *HAL5*), (ii) salt toxicity targets (*HAL2/MET22*, *URA5*), (iii) genes involve in osmolyte synthesis, especially in uptake and export of glycerol (*GPD1*, *GPD2*, *GPP1*, *GPP2*, *FSP1*, *HOG1*) and (iv) genes related to general stress response (*TPS1*, *TPS2*, *SOD1*, *HSP104*). For 16 mutant strains the HR values did not deviate significantly from 1.0 and the effect of gene on heterogeneity was not marked, even if some of the mutants showed net sensitivity to salt (*trk1Δ*, *hog1Δ*) (table 1).

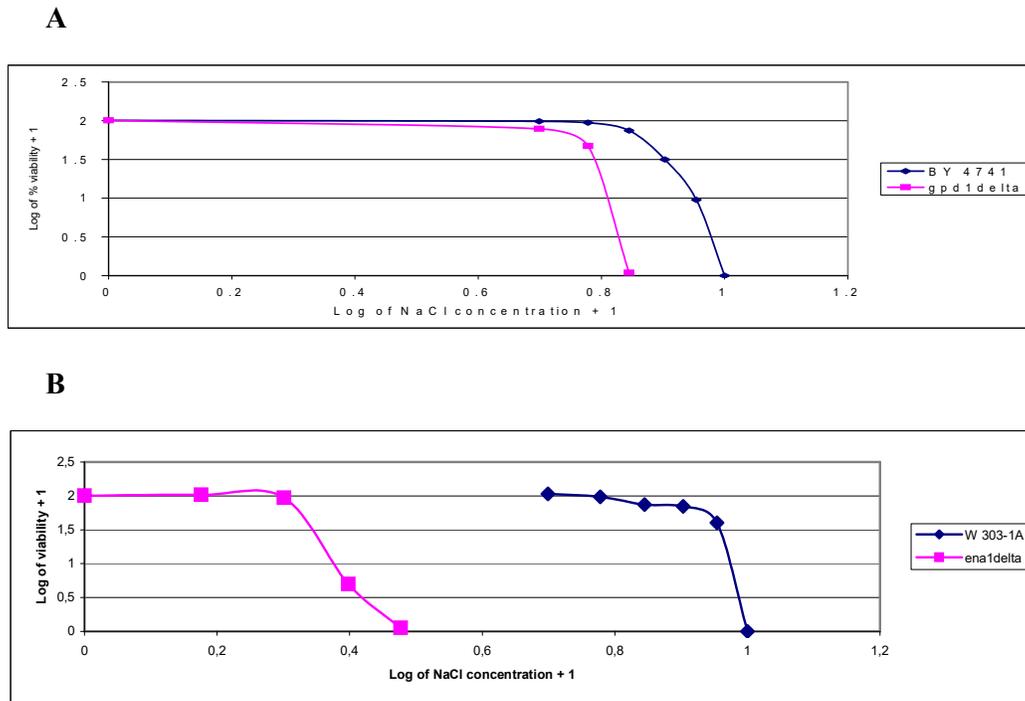
**Table 1.** Screening of the microbial genes join with heterogeneous resistance to salt stress in yeast

<i>Gene name</i>	<i>Function/Mechanism</i>	<i>HR</i>
<b>Ion transporters</b>		
TRK 1, TRK 2	Partially redundant genes encode high-affinity K <sup>+</sup> transporter	1.5 2, 1.57
ENA 1	Li <sup>+</sup> and Na <sup>+</sup> extrusion P-ATP-ase located at plasma membrane	1.9 9
HAL 1,	Activator of TRk 1,2p	1.1 9
HAL 3	Inhibitor of Trk 1,2p	1.2 5
HAL 4/5	Redundant protein kinases activating TRK 1,2p	1.0 3
<b>Salt toxicity targets</b>		
URA 5	Transferase involved in uracil biosynthesis	0.9 3
HAL 2/MET 22	Li <sup>+</sup> and Na <sup>+</sup> -sensitive nucleotidase involved in methionine biosynthesis	1.1 1
<b>Osmolyte synthesis</b>		
GPD 1, GPD 2	Isogenes that encode enzymes involve in the first step of the glycerol metabolic pathway	0.8 2, 1.57
GPP 1, GPP 2	Genes encode glycerol-3-phosphatase (isoforms) that catalyse the second step of the glycerol biosynthesis	1.0 4, 1.25
HOG 1	Hog1 MAP kinase involve in HOG response pathway	0.9 3
FSP 1	Protein mediates both the uptake and efflux of glycerol	0.9 8
<b>General stress response</b>		
TPS 1, TPS 2	Enzymes involve in trehalose biosynthesis, a general stress protectant	0.9 8, 1.0
HSP 104	General stress-responsive gene that encode an ATP-ase driven protein refolding	1.1 3
SOD 1	Cu, Zn superoxid dismutase	1.1 5

*Gpd1Δ* mutant cells were specifically affected for growth at high osmolarity and the phenotype needed to be monitored on plates with 6% salt. Moreover, the dose-response curve for mutant lacking *Gpd1p* (glycerol 3-phosphate dehydrogenase) consistently showed diminished heterogeneity and HR value of ~ 0.82 (figure 1). Expression of *GPD1* is stimulated under various stress conditions: heat shock, oxidative stress, especially under hyperosmotic stress and it is highly dependent on the HOG pathway [10]. The highly homologous isogenes *GPD1* and *GPD2* encode the isoforms of two enzymes that catalyze the first step in glycerol production from glycolytic intermediate dihydroxyacetonephosphate [8].

Mutants lacking the sodium pump encoding *ENA1* gene are highly sensitive to even low concentrations of Na<sup>+</sup> but they do not display osmosensitivity [6]. The cells deficient in *ENA1p* (P-type ATP-ase that mediates the active efflux of sodium ions from cytosol to the exterior) were more heterogeneous and exhibited a significant increase in the degree of cell-

to-cell variability in NaCl resistance compared to isogenic wild-type cells, strain W 303-1A (figure 1). The gradient of viability versus NaCl concentration curve was less steep for *ena1Δ* strain than for wild type and HR ~ 1.99. *ENAI* is part of a gene cluster whose number of repeats is strain specific and its expression is controlled in a highly complex manner by glucose repression, calcineurin and the HOG pathway [6].



**Figure 1.** Salt tolerance of wild type *S. cerevisiae* BY 4741(A), W 303-1A (B) and isogenic mutants YDL022W *gpd1Δ* (A), G 1.9 *ena1Δ* (B).

Long-term adaptation is a complex and additive process, a function of both initial (short-term) survival and an adaptive response (long-term adaptation). In the particular case of saline stress response in yeast, short-term survival is connected with hyperosmotic shock response, especially with production and accumulation of glycerol. Cells initially accumulate glycerol to compensate for differences between the extracellular and intracellular water potential, as osmolyte and osmoprotectant. In fully adapted cells, when cells have reached the final internal glycerol level, this osmolyte has two very different roles, in osmoregulation and in redox-balance. Moreover, it has been shown that of all known compatible solutes, glycerol is the simplest and cheapest to produce and its solubility in water has no limits (“glycerol may be regarded as God’s gift to solute-stressed eukaryotes”) [5]. In this respect it can be expected that *GPD1*, the crucial enzyme in glycerol pathway, stimulated by various stresses, most prominently under osmotic stress, contribute to the cell-to-cell variability in resistance to salt. Using a similar approach, Sumner & Avery proved that *SOD1p* and *CUP1p* contribute to heterogeneous resistance in short-term (10 minutes) copper exposure [17].

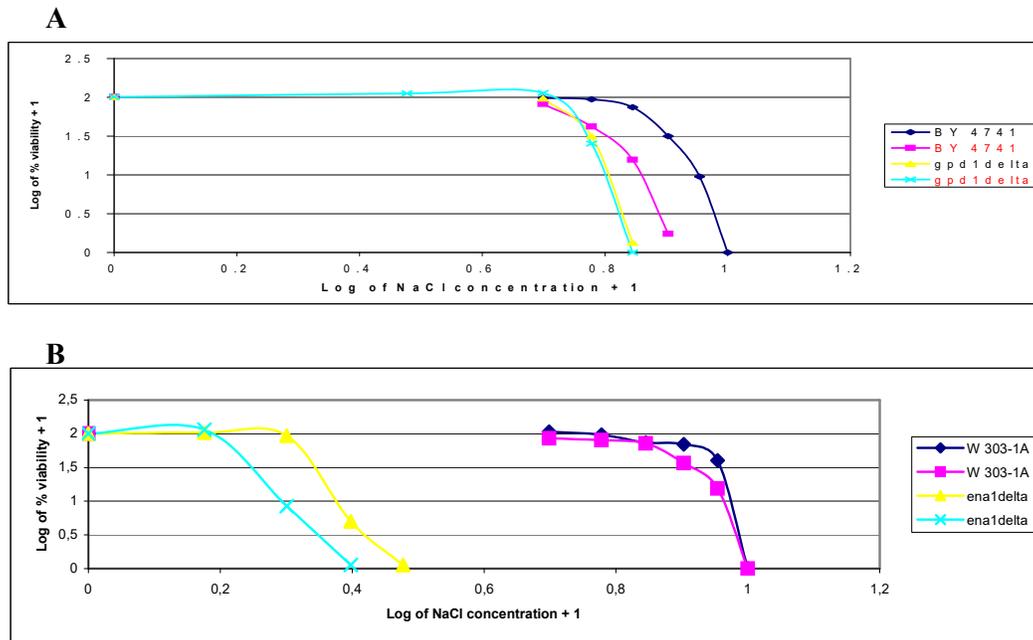
The adaptive response to salt stress is in correlation with ion homeostasis, especially for  $\text{Na}^+$  and  $\text{K}^+$ . A low  $\text{Na}^+$  to  $\text{K}^+$  ratio is essential for salt tolerance. Mutants that failed to maintain low sodium concentration in cell (*ena1Δ*) are very salt sensitive despite normal glycerol accumulation [7]. *ENAI* is induced by starvation, high pH and osmotic stress and our results indicated that this gene acts to buffer heterogeneity in normal conditions.

### Cell cycle progression and heterogeneous salt resistance

Cell cycle progression, cell ageing and mitochondrial activity seem to be dominant factors that drive phenotypic heterogeneity [16].

In order to test the effect of cell cycle stage on salt resistance we compared asynchronous yeast cultures with nocodazole-synchronized cultures for both mutant (*gpd1Δ*, *ena1Δ*) and wild-type (BY 4741, W 303-1A). We proposed that if cell-cycle-arrest treatment (nocodazole arrest cells in G<sub>2</sub>/M phase) diminishes heterogeneity in wild-type cultures this would be reflected in a steeper dose-response curve than in non-treated cultures [17].

In the case of *S. cerevisiae* BY 4741 the nocodazole-arrested culture did not appear to give a steeper dose-response curve than control asynchronous culture. On the other hand, the curve for nocodazole-arrested cells was shifted to the left, indicating greater NaCl-sensitivity of G<sub>2</sub>/M phase cells. Moreover, the dose-response curves for synchronous and asynchronous *gpd1Δ* populations were almost identical (figure 2). In contrast, the salt resistance in control and nocodazole-arrested (G<sub>2</sub>/M-phase) cultures of strain G 1.9 were similar and there was no discernible difference in the gradients of dose-response curves.



**Figure 2.** Influence of cell cycle arrest on salt resistance for wild-type *S. cerevisiae* BY 4741(A), W 303-1A (B) and isogenic mutants YDL022W *gpd 1Δ* (A), G 1.9 *ena 1Δ* (B), asynchronous (black) and nocodazole-arrested (red) cultures

Our results suggested that cell cycle progression could be associated with different salt resistance and that cell cycle-dependent NaCl resistance might be removed in *gpd1Δ* cells. Other studies indicated that accumulation of trehalose, the osmolyte that protect yeast cells against severe osmotic stress, is cell cycle regulated and occurs mainly during G<sub>1</sub> phase [15].

### Osmotic shock and phenotypic diversity

In the first part of this study we analysed the heterogeneous adaptability of cells to saline stress, which encompasses both long-term and short-term responses.

In order to test the genes that modulate the heterogeneous resistance to osmotic shock (short-term stress) we used different techniques, plate count assay, epifluorescence microscopy and flow cytometry. We analyzed the effect of different salt concentrations (0 – 30% [w/v] NaCl) on viability in 10 minutes exposure to salt, using different mutants deficient

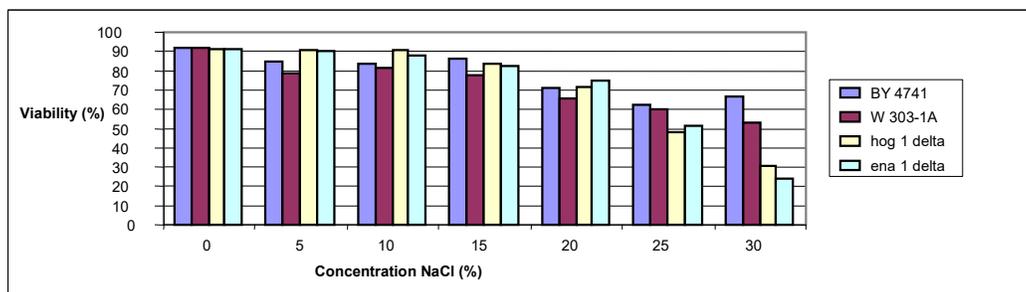
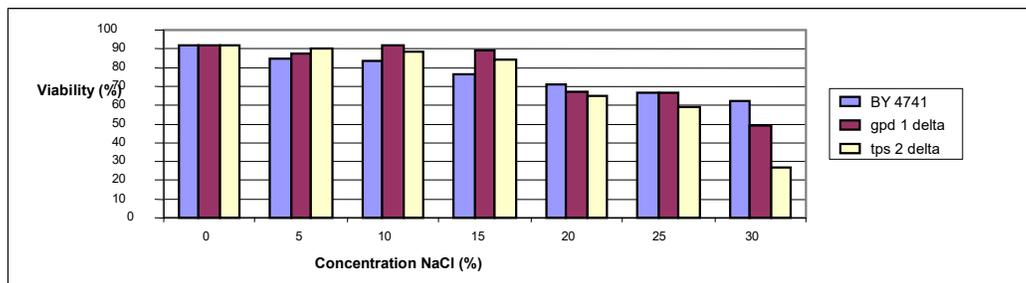
in genes known to respond to osmotic shock (*gpd1Δ*, *hog1Δ*, *tps2Δ*, *ena1Δ*) and two different wild-type strains (BY 4741 and W 303-1A).

The PI viability versus NaCl concentration curves obtained by flow cytometry, similar with those obtained by fluorescence microscopy (standard error between techniques ± 10%), did not show differences between wild and mutants strains, such as were observed in long-term experiments (table 2, figure 3).

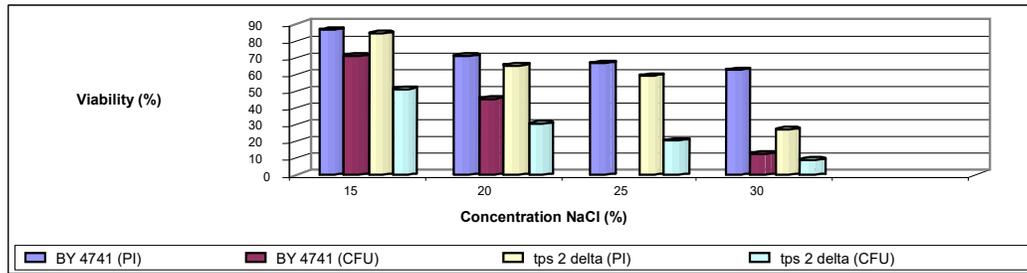
**Table 2.** Fluorescence microscopy evaluation of hyperosmotic shock – PI exclusion test after 10 minutes exposure to different salt concentrations

<i>Saccharomyces cerevisiae</i> strain	Viability on YPG-NaCl 10% medium	Viability on YPG-NaCl 20% medium	Viability on YPG-NaCl 30% medium
BY 4741	84,24 %	69,49 %	52,95 %
W 303-1A	79,10 %	69,85 %	53,66 %
YDL022 W ( <i>gpd1Δ</i> )	89,72 %	70,72 %	41,25 %
YDR074 W ( <i>tps2Δ</i> )	92,45 %	53,84 %	33,56 %

But salt stress proved not to be a binary phenomenon that produces killing in short-term exposure, i.e. after an osmotic shock different phenotypes could be observed: live, live but non-cultivable and dead cells. A hyperosmotic shock causes an instant and rapid loss of water and cell shrinkage but yeast cells placed in high NaCl do not actually die until 2h [10]. Cell proliferation of survivals resumes after about 90 to 120 minutes at about the same point that maximal glycerol levels are achieved. In this case there was a discrepancy between the results obtained by PI fluorescence techniques and plate count assay for determining of salt resistance. It is apparent that, although the majority of cells become non-cultivable after 10 minutes exposure to severe salt stress, many were still able to exclude PI and appear alive with fluorescence techniques (figure 4).



**Figure 3.** Cytometric evaluation of hyperosmotic shock. PI viability at different salt concentrations (0 – 30% [w/v] NaCl) for wild strains *S. cerevisiae* BY 4741, W 303-1A and isogenic strains YDL022W (*gpd1Δ*), YDR074W (*tps2Δ*), YLR113W (*hog1Δ*) and G 1.9 (*ena1Δ*) after 10 minutes exposure to salt stress



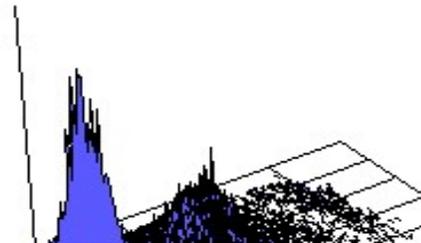
**Figure 4.** Comparison between PI viability obtained by flow cytometry and plate count assay (CFU) for wild strain *S. cerevisiae* BY 4741 and isogenic strain YDR074W (*tps2Δ*).

A hyperosmotic shock causes an instant and rapid loss of water and cell shrinkage. Measurements using Coulter counter indicated that after an osmotic shock with 7% NaCl the cell shrinks to about 30 –35% of its original volume and during the subsequent recovery period the cells increase their volume but they do not reach their initial volume [4]. In mild and severe salt stress shrinking is greater in *gpd1Δ* and *tps2Δ* cells, that are more NaCl sensitive than wild-type strains, proving the protective role of glycerol and trehalose for plasmatic membrane (glycerol plays an important role in survival and adaptation of *Saccharomyces cerevisiae* to moderate osmotic stress, but trehalose is required in severe hyperosmotic shock). The shrink cells were identified on forward scatter and PMT 1 dot plots. The percentage of cells that shrink at 5% NaCl in cells deficient in *GPD1* gene is 10-fold higher compared to wild-type cultures (BY 4741 and W 303-1A). The percentage of cells that shrink at 20% NaCl is almost similar in cells deficient in *TPS2* gene (65.24%) and wild-type cultures BY 4741 (70.97%), but at 30% NaCl is 2.5-fold higher compared to situation at 20% salt (26,96% for YDR074W *tps2Δ* and 66.68% for BY 4741) - figure 5.

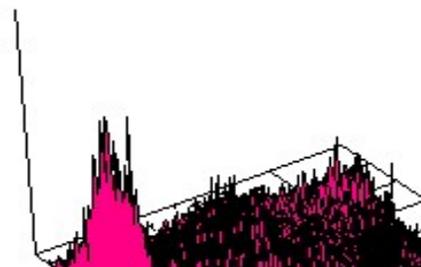
**A**



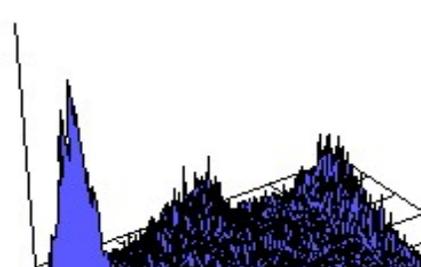
**D**



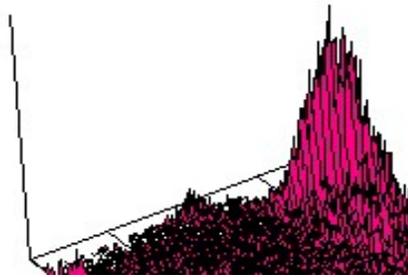
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**E**

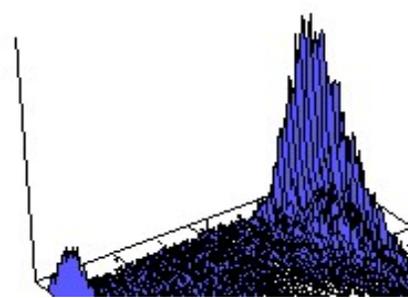


C



**Figure 5A.** Contour plot analysis for wild-typed *S. cerevisiae* BY 4741 after 10 minutes salt stress on YPG-NaCl 10% (A), 20 % (B) and 30% (C) media (axis X FS Lin 1024 FS Lin, axis Y PMT4 Log 1024 PMT4)

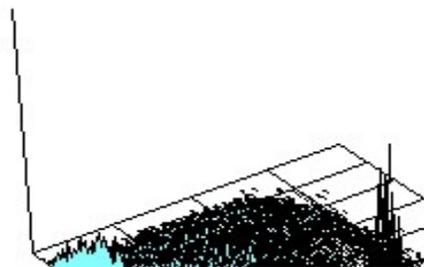
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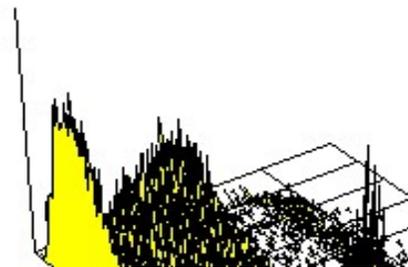
**Figure 5B.** Contour plot analysis for isogenic strain YDR074W (*tps2Δ*) after 10 minutes salt stress on YPG-NaCl 10% (D), 20 % (E) and 30% (F) media (axis X FS Lin 1024 FS Lin, axis Y PMT4 Log 1024 PMT4)

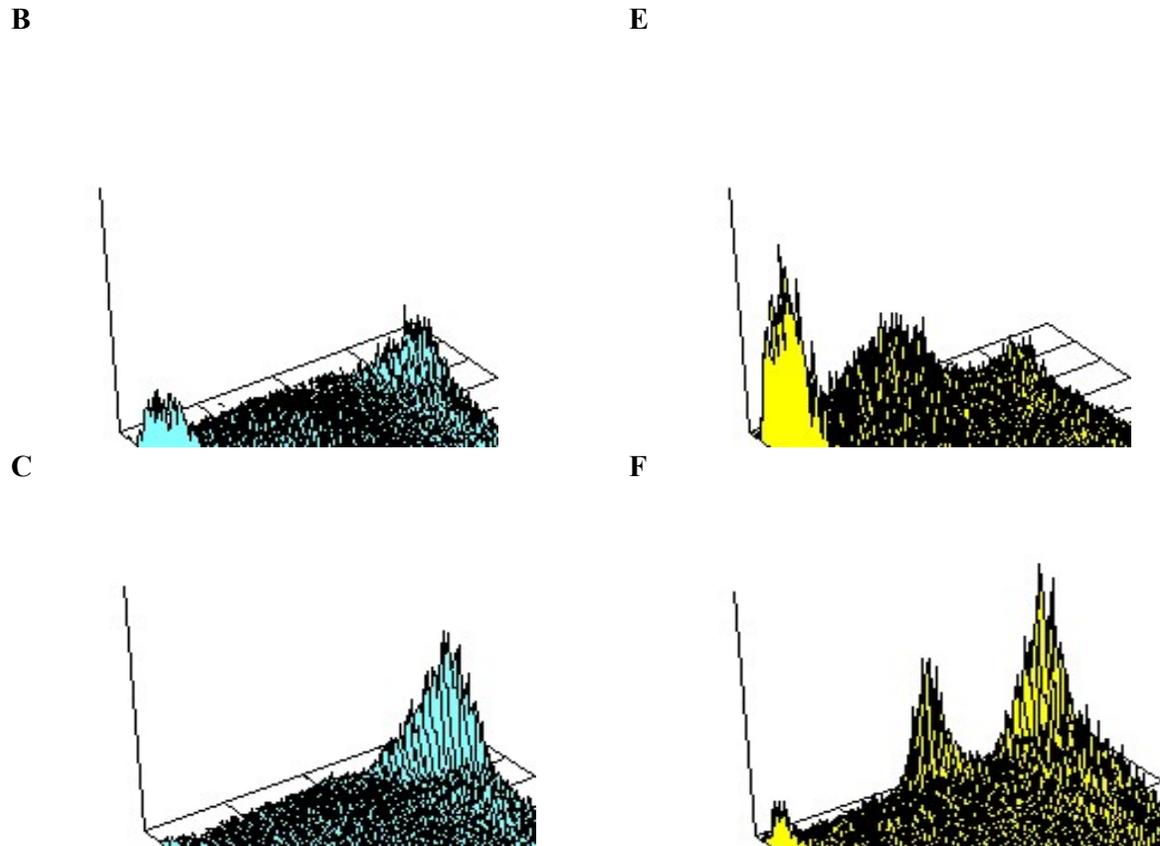
The mutant YLR113W (*hog1Δ*) is more tolerant to short-term salt stress than YDL022W (*gpd1Δ*) and just half of cells shrink at 5% NaCl compared to *gpd1Δ* (data not shown). This relative resistance of *hog1Δ* strain indicated that there is in *Saccharomyces* sp. a significant HOG-independent response. Hohmann S. et al. showed that *hog1Δ* cells accumulate only half amount of glycerol than wild-type under hyperosmotic stress, but the signalling pathway(s) for glycerol production in this case remain to be identified [7]. The strain G 1.9 has a shrinkage process similar with those from wild types *S. cerevisiae* W 303-1A and BY 4741 (figure 6).

A



D





**Figure 6A.** Contour plot analysis for wild strain *S. cerevisiae* W 303-1A after 10 minutes salt stress on YPG-NaCl 10% (A), 20 % (B) and 30% (C) media (axis X FS Lin 1024 FS Lin, axis Y PMT4 Log 1024 PMT4)

**Figure 6B.** Contour plot analysis for isogenic strain G 1.9 (*ena1Δ*) after 10 minutes salt stress on YPG-NaCl 10% (D), 20 % (E) and 30% (F) media (axis X FS Lin 1024 FS Lin, axis Y PMT4 Log 1024 PMT)

## Conclusions

In conclusion, after the preliminary screening the activity of two genes was correlated with the heterogeneous resistance to long-term salt stress response in yeast, *GPD1* gene contribute to heterogeneity and *ENAI* maybe in a gene network that masks, or buffers, heterogeneity in the wild *Saccharomyces cerevisiae*. The *GPD1* may help to establish the cell cycle-dependency of heterogeneous salt resistance. Further studies could focus on the stress response at single cell level using flow cytometry and GFP technology and to comparative study on the heterogeneity in expression of *GPD 1* gene and a constitutive gene.

Using different techniques (flow cytometry, fluorescence microscopy and plate count assay) we proved that hyperosmotic shock produces non-cultivable cells and that the shrinking process is more marked in mutant *gpd1Δ* at mild stress and in mutant *tps2Δ* at severe salt stress. The experiments proved not to be altered heterogeneity in short term NaCl stress for all the tested mutants.

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