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Eglė LASTAUSKIENĖ

INFLUENCE OF THE MEMBERS OF RAS/PKA SIGNAL TRANSDUCTION PATHWAY ON THE YEAST *SACCHAROMYCES CEREVISIAE* CELL DEATH INDUCED BY NATURAL ACIDIFICATION OF THE MEDIUM

Summary of doctoral dissertation

Biomedical Sciences, Biology (01B)

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This dissertation was prepared 2004-2008 year at the Department of Microbiology and Biotechnology, Faculty of Natural Sciences of Vilnius University.

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VILNIAUS UNIVERSITETAS

Eglė LASTAUSKIENĖ

RAS/PKA SIGNALINIO KELIO KOMPONENTŲ ĮTAKA NATŪRALIU TERPĖS RŪGŠTĖJIMU INDUKUOJAMAI *SACCHAROMYCES CEREVISIAE* LĄSTELIŲ ŽŪČIAI

Daktaro disertacijos santrauka

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Disertacija bus ginama viešame Biologijos mokslo krypties tarybos posėdyje 2011 m. gegužės 11 d., 15 valandą Vilniaus universiteto Gamtos mokslų fakulteto prof. Jono Dagio auditorijoje.

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Disertaciją galima peržiūrėti Vilniaus universiteto biobliotekoje.

INTRODUCTION

Constantly changing environment is the major factor controlling the growth and development of the microorganisms. For quick generation of the cell response, information about changes in the cell environment is rapidly transmitted to the inner molecules of the cell. During the course of evolution the cells have developed signaling systems that are able to combine extracellular signals with the inner processes, as transcription, translation etc. One of the universal signaling systems is Ras/PKA signal transduction pathway (Estruch 2000). This system helps cells recognize the nutrient sources present in the growth medium. Ras/PKA signal transduction pathway controls variety of the cell functions: cell proliferation, sporulation, aging, cell death, response to stress and others (Thevelein, de Winde, 1999). This pathway is conserved among eukaryotic organisms; even in prokaryotes some components of the pathway can be found. Alterations in the Ras/PKA pathway may strongly decrease cell viability and influence the general homeostasis of the cell. The reduction of the homeostasis may cause cell aging and finally cell death (Thevelein, de Winde, 1999; Verselle et al., 2001). Yeast cell are also able to change their environment through production of metabolites, pheromones and other molecules. Environmental pH is one of the main factors influencing the growth, physiology and differentiation of yeast (Penalva, Arst, 2004). In *Saccharomyces cerevisiae*, response to pH is determined by the RIM101 pathway. Proteolytically processed Rim101p is required for the response to alkaline pH. Extracellular acidification leads to intracellular acidification which causes stress to the yeast cells and activates Ras/PKA signal transduction pathway (Mollapour et al., 2006). Recently, it was shown, that Ras/PKA signal transduction pathway regulates cell aging as response to environmental pH (Burtner et al., 2009).

Many aging and apoptosis features are conserved between yeast and multicellular microorganisms, and this makes them perfect model organisms. Yeasts are also suitable also for acidosis related disease studies.

Yeasts *Saccharomyces cerevisiae* are valuable not only as a model organisms for studying molecular processes of the higher mammals. They are widely used in food and alcohol industry. Growth of the yeast cells are followed by an acidification of the medium. In some branches of industry such acidification is highly welcome in other –

totally undesirable. It is known, that quality of the sparkling wines is basically related to acidification of the surrounding medium and autolysis of the cells (Tabera et al., 2006). Autolysis of the yeast cells is the main source of molecules such as peptides and amino acids. Ras/PKA signal transduction pathway is able to control acidification of the medium and also selection of the death types of the cell. Ability to control these processes will allow to extend the use of yeast in the food and alcohol production.

The aim of the dissertation work was: to determine the influence of the members of Ras/PKA signal transduction pathway on the selection of cell death program in yeast *Saccharomyces cerevisiae* caused by the natural gradual acidification of the medium.

The following **tasks** have been formulated:

1. To create model system for analysis of influence of Ras/PKA signal transduction pathway members on the yeast cell death in naturally (during cell growth) acidified medium and under acid stress conditions.
2. To evaluate influence of the members of Ras/PKA signal transduction pathway on the cell viability of isogenic strains under naturally (during cell growth) changing pH conditions (SC medium) and in constant pH conditions (SC-MES medium).
3. To evaluate influence of the members of Ras/PKA signal transduction pathway on the selection of cell death program – apoptosis, necrosis.

Scientific novelty

The novelty of this research consist of systemic analysis of the influence of members of Ras/PKA signal transduction pathway on the yeast *Saccharomyces cerevisiae* cell death induced by natural gradual acidification of the growth medium.

- Influence of the members of Ras/PKA signal transduction pathway on the acidification of the medium was determined. Deletion of the *RAS2* gene, causing downregulation of activity of the pathway, or inactivation of *PDE1* increases acidification of the medium as compared to the wild type strain.
- For the first time evaluation of the influence of Ras/PKA pathway on the cell viability in naturally acidified medium was performed. We showed, that cell

viability is related to mutations in the genes of members of Ras/PKA signal transduction pathway: under gradual acidification conditions cell viability of *Ras2*^{Val19} and *ΔPDE2* strains is significantly lower as compared to wild type strain. Therefore deletion of the *RAS* genes and inactivation of the phosphodiesterase1 caused significant increase in cell viability.

- Influence of Ras/PKA and changing environmental pH on the cell aging was determined. We showed that buffering of the medium increases cell growth speed and extend cell age. Decrease of the Ras/PKA activity increases lifespan of the yeast.
- It was shown that during the natural acidification Ras/PKA signal transduction pathway regulates selection of the cell death program. In this process *RAS2* gene acts as an inducer of apoptosis, while *PDE2* acts as inducer of necrosis.

These results allow extending the appliance of yeast as a model organism in the studies of cell aging and death of higher eukaryotes. The ability to control cell death using the strains with altered Ras/PKA activity will be beneficial to biotechnology.

Content of the dissertation. Dissertation is written in Lithuanian language. Dissertation consists of background, material and methods, results, discussion, conclusions and references (201 positions). In the dissertation there are 8 tables and 38 figures. In total 136 pages.

MATERIAL AND METHODS

Yeast strains. SP1 MATα *his3 leu2 ura3 trp1 ade8 can*^{R}, **TK161R2V** MATα *his3 leu2 ura3 trp1 ade8 can*^{R} *Ras2*^{Val19}, **SP1 *ras1Δras2Δ/p21*** MATα *his3 leu2 ura3 trp1 ade8 can*^{R} *ras2::LEU2, ras1::URA3 (pHa-ras, TRP1),* **SP1 *ras1Δ*** MATα *his3 leu2 ura3 trp1 ade8 can*^{R} *ras1:: URA3,* **SP1 *ras2Δ*** MATα *his3 leu2 ura3 trp1 ade8 can*^{R} *ras2::LEU2* (Stanhill et al., 1999) kind gift of prof. D. Engelberg, University of Jerusalem.
Tr1 SP1 *ras1Δ [pJR1859],* **Tr2** SP1 *ras2Δ [pJR1244],* **Tr3** SP1 *ras2*^{Val19} *[pJR1052],* ***Tr4*** *MATa his3 leu2 ura3 trp1 ade8 can*^{R} *pde2::HIS3 (YCpPDE2(URA3)),* ***Tr5*** *MATa his3 leu2 ura3 trp1 ade8 can*^{R} *pde1::HIS3 (YCpPDE1(LEU2))* (This work).
JT134 *MATa his3 leu2 ura3 trp1 ade8 can*^{R} *pde2::HIS3,* ***JT135*** *MATa his3 leu2 ura3 trp1 ade8 can*^{R} *pde1::LEU2,* ***JT136*** *MATa his3 leu2 ura3 trp1 ade8 can*^{R} *pde1::URA3 pde2::HIS3,* ***JT21569*** *MATa his3 leu2 ura3 trp1 ade8 can*^{R} *tpk1*^{w1} *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 + YCpU-PDE1,* ***JT21570*** *MATa his3 leu2 ura3 trp1 ade8 can*^{R} *tpk1*^{w1} *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 + YCpPDE2(URA3)* (Ma et al., 1999) Kindly provided by Prof. J. M. Thevelein, Katholieke Universiteit, Leuven.

Plasmids. JR1052, pJR1059, pJR1244 were provided by Prof. J. Rine (Suter et al., 2004). YCpPDE1, YCpPDE2 were a gift of Prof. J. M. Thevelein (Ma et al., 1999). Yeast cells were transformed as described in Gietz et al., 1995, or performing electroporation as described in http://www.uhmc.sunysb.edu/bioscience/default.htm.

Growth media and cultivation conditions. Yeast were grown in YPD medium, containing 2 % glucose, 2 % peptone, 1 % yeast extract and in SC medium, containing 6.7 g/l yeast nitrogen base (w/o amino acids, with ammonium sulfate), 2 % glucose and supplemented with the appropriate amino acids. When the acidification had to be eliminated the growth media were buffered using 2-morpholinoethanesulfonic acid (MES). Starting pH of the media in all cases was 6.2 for YPD and 5.4 for SC medium. For the acid stress inductions sorbiltol 1M solution was titrated to pH 5.4 and pH 2.1 using HCl and NaOH.

Analysis of the altered Ras/PKA signal transduction pathways activity phenotypes.
For the detection of altered Ras/PKA activity phenotypes standard experiments were performed. Survival and growth on the medium with different carbon source analysis was tested using YPD, YPA, YPG, YPGal and N^- media as described in Toda et al. 1985.
Glycogen accumulation was evaluated by staining yeasts with aquatic iodine solution, analyzing intensity of the color after 1 minute treatment.
Heat shock was performed as described in Broek et al., 1987, with a few modifications: yeast cells were incubated at 55 ºC for 10, 20 and 30 minutes. After the heat treatment cells were plated on the new media and incubated at 30 ºC.

Evaluation of the cell viability. For the cell viability assay, yeast strains were grown for 78 hours in the liquid YPD, YPD-MES, SC, SC-MES media. Samples for the viability measurement were taken at 22nd (exponential phase), 46th (early stationary phase) and 72nd hour of growth (late stationary phase).

For the microscopy analysis, yeast cells were stained with 50 µM propidium iodide (PI) and immediately analyzed by fluorescent microscopy, 560 nm wavelength (Olympus Provis AX70TRF microscope).
For the flow cytometry analysis, 0.5 ml 50mM Na citrate containing 8 µl/ml PI was added to the harvested yeast cells. Cells were processed immediately. For each sample 20000 cells were counted. Yeast cell were analyzed in Institution of Immunology by using „FACSort“ flow-cytometer.
For evaluation of the colony formation abilities, yeast cells were grown in liquid media; after 72 hours of growth, cell number was counted using a Bürker chamber. 100 µl of known amount of the cells were plated on the YPD medium. Formed colonies were counted after 3 days of incubation at 30 °C.

Induction of acid stress. For the acid stress induction, yeast cells were grown in the YPD medium for 26 hours till the exponential stage, harvested and incubated in two solutions – pH 5.4 and pH 2.1 – for 4 and 6 hours. After the incubation, viability of the cells or detection of the apoptotic markers was evaluated using microscopy, flow-cytometry or colony formation ability test (as described above).

Detection of the early apoptotic markers. For DAPI staining 4µl of solution containing 5% DAPI dye was added to the immobilized cells. Samples were immediately analyzed by fluorescent microscopy using Nikon eclipse 80i microscope or performing confocal microscopy using NIKON Eclipse TE2000-S, C1plus at the Institute of Biochemistry.

TUNEL staining was performed using Calbiochem Fluorescein FregEL DNA Fragmentation Detection Kit following manufacturers recommendations as described in Madeo et al., 1997. For the permeabilisation of speciment, proteinase K was used.

For the phosphatidylserine exposure detection yeast cells were stained using Annexin-V-Fluos (FITC coupled annexin) and propidium iodide as described Madeo et al., 1997. After the staining yeast cell were analyzed by fluorescent microscopy and flow cytometry.

Active caspase detection was carried-out using CaspACE™ FITC-VAD-FMK *in situ* marker, Promega, and following manufacturers recommendations. After the staining cells were analyzed by fluorescent microscopy.
For each microscopy sample about 300 cells were analyzed. During flow cytometry procedure for each sample 20000 cells were counted. All experiments were independently repeated 3 times.

RESULTS

1. Evaluation of the activity of Ras/PKA pathway in analyzed strains.

Activity of the Ras/PKA signal transduction pathway can be evaluated by performing classic genetic experiments (Toda et al., 1995). For this purpose we tested resistance of the yeast cells to the heat shock, amount of the accumulated glycogen, and ability to grow (30 ºC) and survive (37 ºC) on the medium with nonfermentable of poorly fermentable carbon source. After the summarizing results we divided analyzed strains into 3 groups in relation to the Ras/PKA activity: 1. Yeast strains with normal activity of Ras/PKA signal transduction pathway – SP, *ΔPDE1, ΔPDE1/2, Tr1, Tr2, Tr3, Tr4, and Tr5*. 2. Yeast strains with increased Ras/PKA activity - *Ras2*^{Val19}, *PDE2* and *JT21569.* 3. Yeast strains with reduced Ras/PKA activity - *ΔRas1, ΔRas2, ΔRas1/2, JT21570.*

2. Influence of the members of Ras/PKA signal transduction pathway on the gradual acidification of medium and growth rate of yeast cell

For evaluation of metabolic activity and cell growth rate yeast strains were grown in two media: YPD and SC. Every 6 hours media pH and OD of the culture was measured. Starting pH for YPD medium was 6.2, and for SC medium 5.4. To retain constant pH we used MES buffer.

During yeast cell growth in an YPD medium, independently of the genotype of the strain, only a slight acidification of the medium was observed. Such stability of the medium pH may be caused by a possible buffering effect of peptone. In YPD and YPD-MES medium yeast cells are growing rapidly and reaching high density of the culture. There was no difference between cell growths in YPD or YPD-MES medium, therefore MES buffer was suitable for further experiments.

A significant medium acidification was observed when yeast strains were grown in SC medium. According to the ability to change extracellular pH, yeast strains were divided into two groups. The first group slightly acidified the growth medium. This group contained SP1, *Ras2*^{Val19}, *ΔRas1, ΔPDE2, ΔPDE1/2, JT21569* strains and *Tr1, Tr3, Tr4* and *Tr5* transformants. The second group of strains was determined by a

significant acidification of the medium and increased growth rate of the cells. This group comprised of *ΔRas2, ΔRas1/2, ΔPDE1, JT21570* and *Tr2*. It was shown, that buffering of the SC medium significantly increases cell growth rate.

3. Evaluation of the yeast cell viability during gradual acidification of medium and under acid stress conditions

For the evaluation of cell viability yeast cells were grown in YPD, YPD-MES, SC, SC-MES medium. Samples were taken from the exponential stage of growth (22 h - control), early stationary (46 h) and late stationary (78 h) stage. Yeast cells were stained using propidium iodide and immediately analyzed by fluorescent microscopy, flow-cytometry or ability to produce a colony.

Cultivation of the yeast cells in YPD and YPD-MES media showed, that viability of the cells remains high even in late stationary stage.

The first differences of the cell viability, as compared to the wild type strain, were observed already after 46 h of growth in SC medium. Statistically significant decrease in cell viability was observed in $Ras2^{Val19}$ strain.

After 78h of cultivation in SC medium significant cell viability changes were detected (fig. 1). Cell viability of the wild type strain was 34.17 ± 6.532 %. Viability of the *ΔPDE2*, *Tr4*, *Tr1* and *Tr5* did not differ from SP1. Constitutive activation of the Ras/PKA led to loss of the cell viability under gradual acidification conditions. Only 17.8 ± 2.459 % living cells were counted in $Ras2^{Val19}$ strain after 78 h of growth in SC medium. Significant decrease of cell viability (as compared to SP1) was detected also in *Tr3* and *JT21570* strains. Therefore, increased number of living cells was counted in *ΔRas2, ΔRas1/2, ΔRas1, Tr2*, *ΔPDE1, ΔPDE1/2, JT21570* strains.

Buffering of the SC medium caused significant increase in cell viability. In all the cases cell viability was significantly higher after 78 h of growth in SC medium as compared to SC-MES medium.

These results were also confirmed by flow-cytometry analysis. Generally flow-cytometry results showed 10-20 % lower cell viability as compared to microscopy analysis. These differences can occur because of the sensitivity of the methods. During cell staining with propidium iodide, a different intensity of staining can be observed. In

the microscopy analysis, only completely stained (red) cells are counted as “dead”, while in flow-cytometry even slightly stained (light red) cells or cell fragments are registered as “positive” signal. Regardless of differences between microscopy and flow-cytometry results, the tendency of strain cell viability is the same.

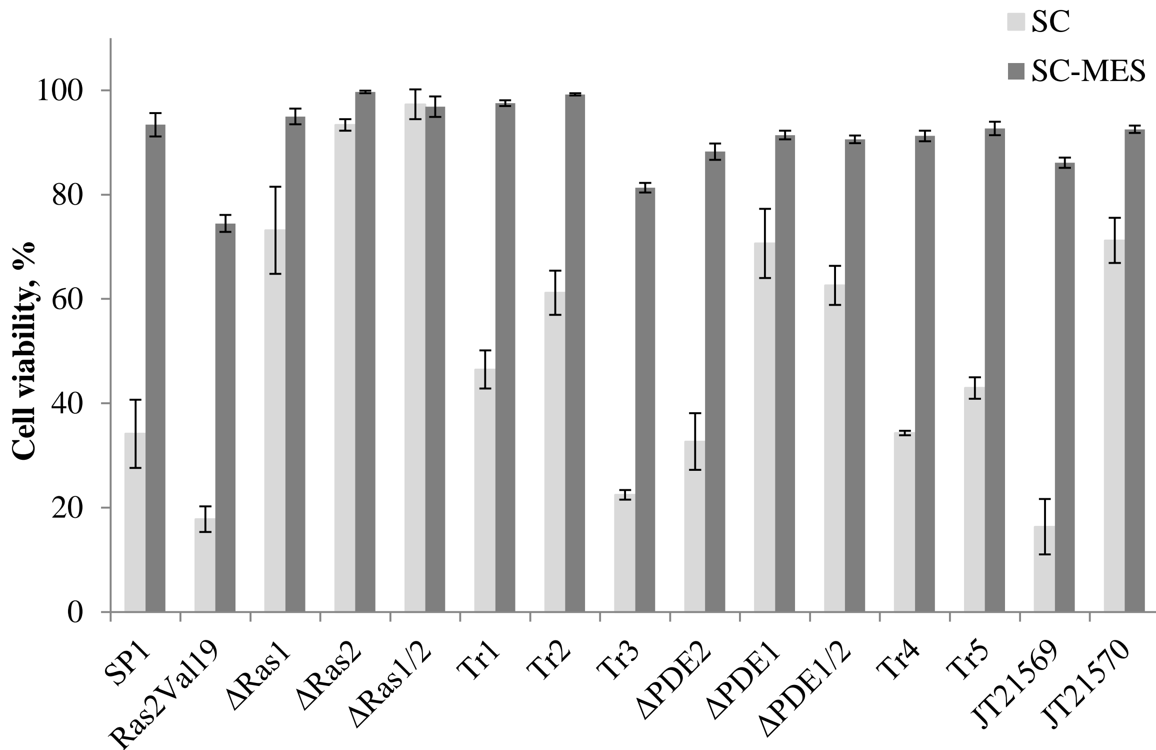

Figure 1. Viability of the cells in the yeast strains with altered Ras/PKA activity after 78 h of growth in SC and SC-MES medium.

For the colony formation a known amount of cells cultivated in different media were placed on the YPD plates and after 48 h number the present colonies were counted. Results of the colony formation test confirmed results of microscopy and flow-cytometry. It was shown that not all the cells counted as living during microscopy analysis are able to retain replicative potential and produce a colony. As in microscopy and flow-cytometry analysis, colony formation test also showed that buffering of the medium significantly increased cell viability.

Analysis of the cell viability showed that mutation in Ras/PKA signal transduction pathway affect yeast cell viability as response to the environmental pH. Ras/PKA signal transduction pathway can act as a positive or negative regulator of the cell viability dependent on the pH of the medium. To confirm this assumption we performed acid stress induction experiments.

For the acid stress analysis we have grown yeast cells in YPD medium till the middle of exponential stage and placed them into two sorbitol solutions of 5.4 and 2.1 pH for 4 h and 6 h. After the incubation cell viability was also tested by microscopy, flow-cytometry and colony formation abilities.

Significant changes in cell viability were detected after cell incubation in 2.1 pH buffer already after 4 h (fig. 2).

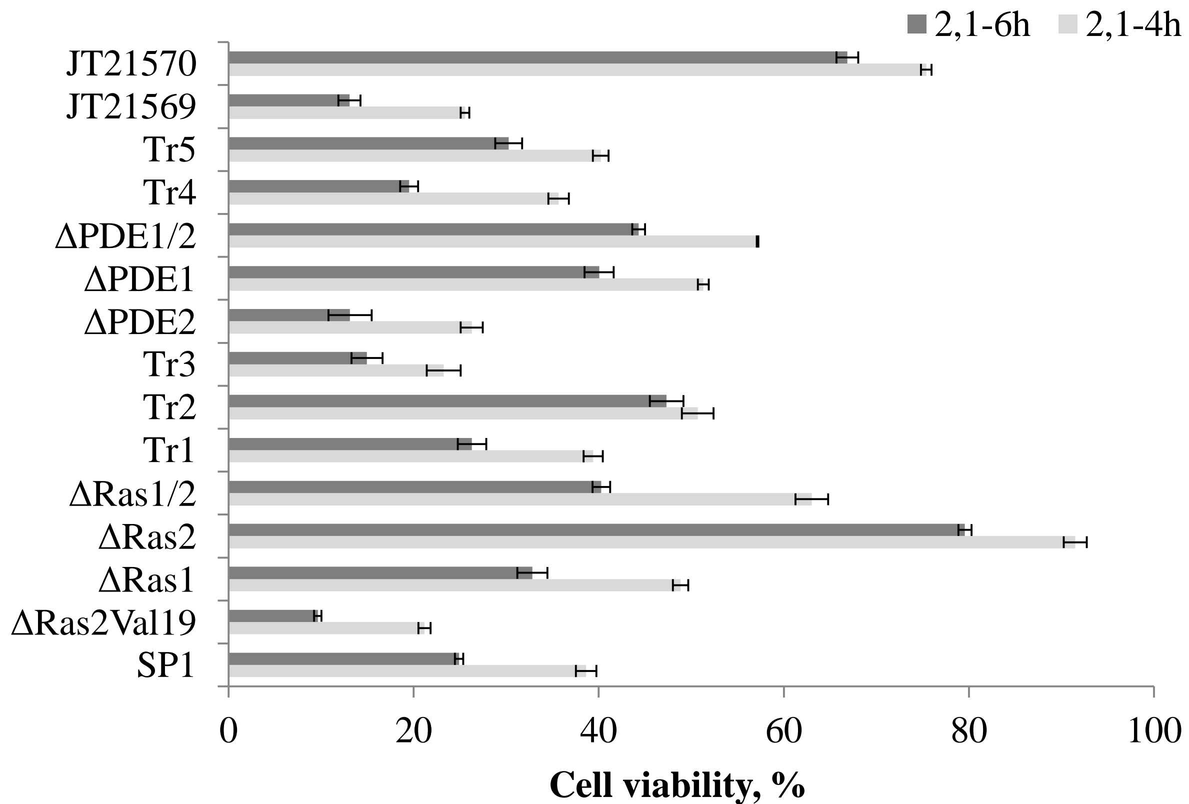

Figure 2. Cell viability of the yeast strains with altered Ras/PKA activity after incubation in 2.1 pH solution for 4 h and 6 h.

Significant decreased of cell viability as compare to SP1 was detected in *Ras2*^{Val19}, *Tr3, ΔPDE2* and *JT21569*. During natural acidification of medium, as well as under acid stress conditions deletion of the *RAS* genes caused increased resistance to the low environmental pH and a significant increase in cell viability (*ΔRas1, ΔRas2, ΔRas1/2)*. Yeast cell transformation with the medium copy number plasmid was not able fully restore wild type phenotype. Viability of the *Tr3* and *Tr2* cells significantly differed from the SP1 and also from the parental strain. Inactivation of the phosphodiesterase 1 increased the cell viability under acid stress conditions. In *ΔPDE1* and *ΔPDE1/2* strains number of the living cells was over 50%. Also introduction of the second copy of *PDE2*

gene to the strain with PKA mutation caused significant increase in cell viability, as compared to SP1.

Prolongation of the incubation time till 6h in all strains caused significant decreased of cell viability.

Microscopy results were also confirmed by flow-cytometry. As previously, flow-cytometry showed generally lower number of living cells in population, but variations of cell viability remained the same.

Acid stress induction also affected yeast replication potential. Colony formation analysis showed that significant decrease in colony production as compared to SP1 was detected in *Ras2*^{Val19}*, Tr3, ΔPDE2, JT21569*. Therefore more colonies were counted in the case of *ΔRas2, ΔRas1/2, Tr2, ΔPDE1, ΔPDE1/2* strains. And number of produced colonies of *ΔRas1, Tr1, Tr4* and *Tr5* strains did not differ from SP1.

Members of the Ras/PKA signal transduction pathway regulate cell viability during gradual acidification of medium, as well as in acid stress conditions. *RAS* genes and phosphodiesterase1 are acting as negative regulators of cell viability in acidic conditions. Phosphodiesterase2 seems to act as a positive regulator in these conditions. Analysis of the cell viability allowed us to evaluate effect of the mutations in Ras/PKA pathway on the survival of yeast cells in the environment created naturally by the cell growth and under acid stress conditions.

4. Detection of early apoptotic phenotypes in yeast strains with altered Ras/PKA activity

Changes in nucleus morphology are one of the early apoptosis markers in yeast cells. These changes were detected by staining yeast cell with DAPI and performing fluorescent or confocal microscopy.

Less than 1 % of cells with nucleus fragmentation were detected in yeast cells after the growth in YPD and YPD-MES media. Therefore after 78 h of growth in SC medium we detected various changes of the nucleus morphology in the yeast cells. There were different structures of the nucleus registered: fragmentation of the nucleus, cells with double nucleus, cells without visible nucleus and others. In *ΔRas2* and *ΔRas1/2* an increase in the cell number (as compared to SP1) with double nucleus was detected.

Appearance of the double nucleus can be related to endomitosis in yeast cells (Pichova et al., 1997; Lun et al., 2001) and also can be a result of the symmetric nucleus fragmentation (Madeo et al., 1997). In this discussion we support theory of endomitosis. The size of the double nucleus is the same as in the cell with one nucleus, and after the fragmentation the size of the two fragments is usually smaller than the normal nucleus. And also the high resistance of the yeast cells to stress conditions can be related to the presence of a double genome in one single cell.

Nucleus morphology examination results do not correlates with the results of cell viability or other markers of early apoptosis. Changes in nucleus structure can only predict possible apoptosis in yeast cell, but are not able to show the real number of the cells dying in the apoptotic way. Part of the changes is not lethal to the cell and can be repaired after the change of growth conditions.

For the further analysis of the apoptosis in the yeast cells during the gradual acidification of medium, as well as under acid stress conditions we performed detection of early apoptotic phenotypes. For this purpose phosphatidylserine detection (annexinV staining), evaluation of DNA fragmentation (TUNEL reaction) and active caspase detection was performed. There was a correlation between 3 different markers of early apoptosis in the yeast cells (fig. 3). In SP1 the results of the phosphatidylserine exposure and activation of caspase were equal. TUNEL assay showed about 10 % less cells with the fragmented DNA as compared to the other markers. In all transformed strains (*Tr1, Tr2, Tr3, Tr4, Tr5*) and also *ΔPDE1* the percentage of the apoptotic cells was statistically similar to SP1.

Statistically significantly lower amount of apoptotic cells was detected in *ΔRas1, ΔRas2, ΔRas1/2* and *JT21570* strains. These results allow us to affirm, that inactivation of the *RAS* genes and also introduction of the v-Ha-ras into yeast cells stops apoptotic program of the cell death in yeast by prolonging the cell's lifespan and inducing necrotic way of cell death. Also we confirm the hypothesis, that introduction of the additional copy of *PDE2* gene can rescue the yeast cells from apoptosis caused, by an inactivation of the PKA (Ma et al., 1999). Therefore the additional copy of the *PDE1* gene does not have such effect; number of the apoptotic cells in *JT21569* was significantly higher as compared to SP1. Deletion of both phosphodiesterase genes (*ΔPDE1/2)* lessened the number of the apoptotic cells.

The highest number of the apoptotic cells was detected in yeast strains with the least cell viability: *Ras2*^{Val19} and *ΔPDE2*. Such high percentage of the apoptotic cells shows, that even in the cells counted as living by microscopy analysis processes of the early apoptosis are already begun.

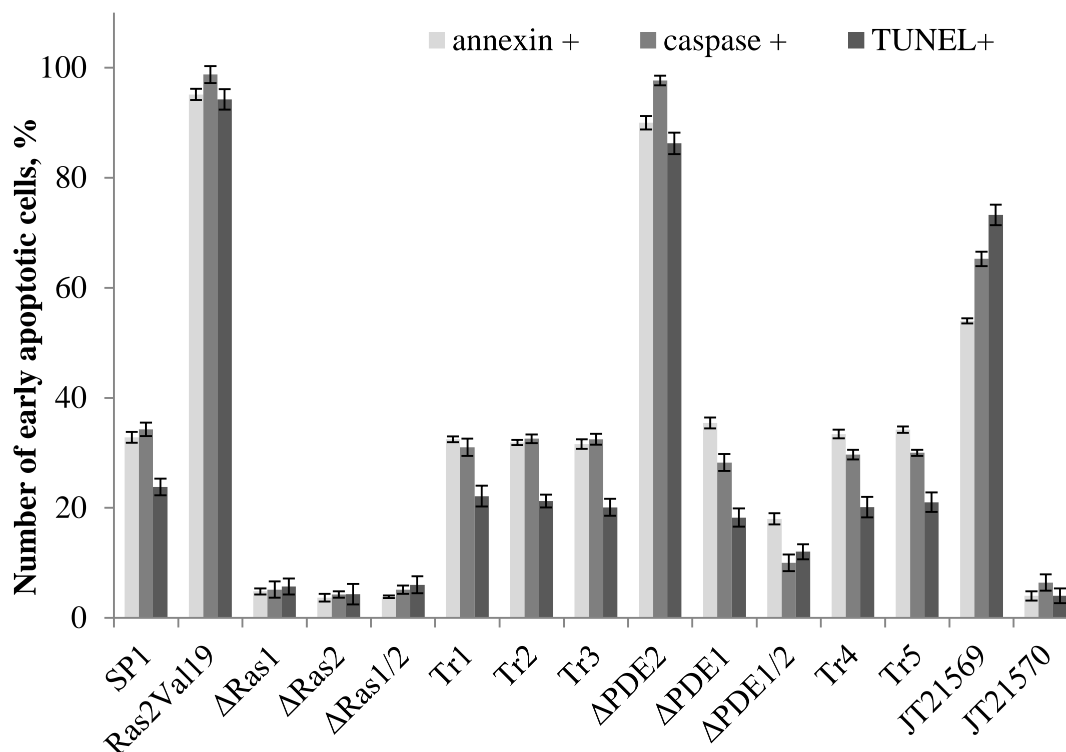

Figure 3. Number of the early apoptotic marker in the yeast cells after 78 h of growth in SC medium.

Number of the apoptotic cells in the population after the cell growth in YPD and YPD-MES medium was less than 1 %. Buffering of the medium increases cell's lifespan and diminishes cell death with the markers of apoptosis. Number of the apoptotic cells in the population after the growth in SC-MES medium was less than 6 %.

Acid stress induction showed similar results (fig. 4). Correlation between three markers of early apoptosis was also determined in the cells after incubation in pH 2.1 for 4 h and 6 h. Yeast strains sensitive to the gradual acidification of the medium showed also high level of the apoptotic marker after the acid shock induction. Significantly more apoptotic cells were detected in *Ras2*^{Val19}, *ΔPDE2* and *JT21569* strains. In all transformed strains and also in *ΔPDE1* number of the cells with active caspase was

similar to SP1. In *ΔRas1, ΔRas2, ΔRas1/2, ΔPDE1/2* and *JT21570* strains the number of the apoptotic cells was significantly less as compare to SP1.
Phosphatidylserine and DNA fragmentation detection results are similar to the results of caspase activation.

In all the cases the incubation of the yeast cells in pH 5.4 solution did not increase the number of the early apoptotic cells.

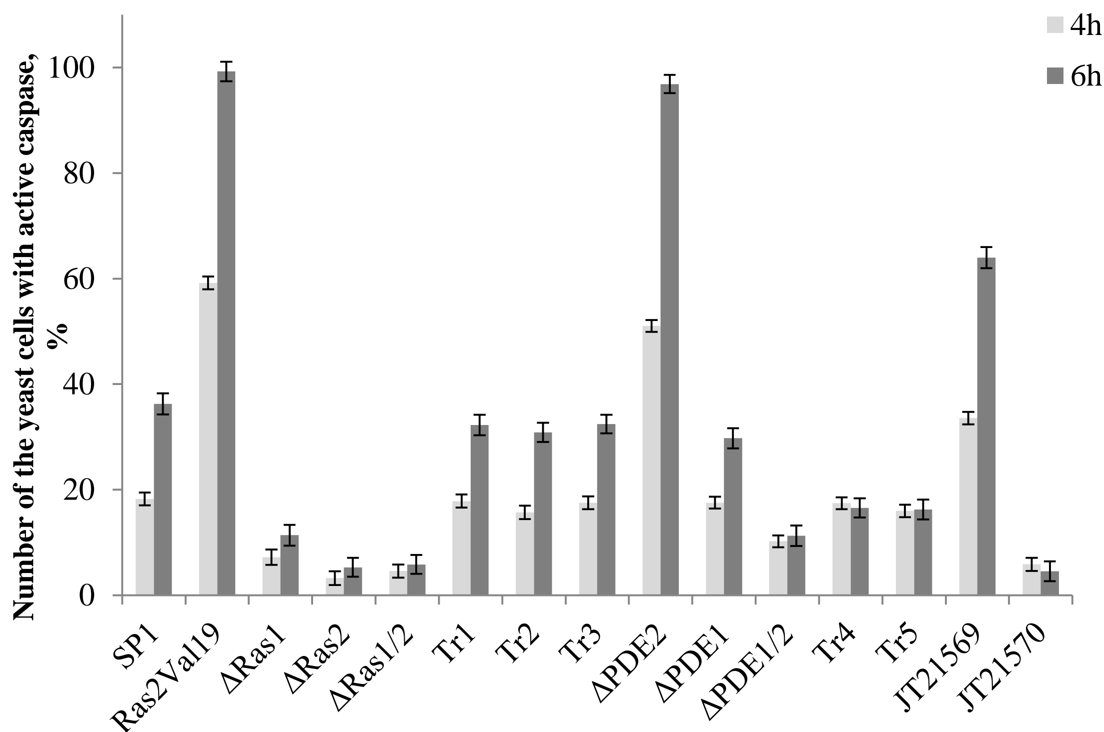

Figure 4. Number of the yeast cells with an activated caspase in the yeast strains after the incubation of cells in pH 2.1 solution for 4 h and 6 h.

Results of the determination of cell death type have proven that members of the Ras/PKA signal transduction pathway regulate the mechanisms of cell aging and death in yeast cells. Possibility to control these processes will extend field of appliance of such strains as a model organisms for studying of human aging and cell death and also in food industry.

DISCUSSION

Microorganisms and environment create united interacting system. Constantly changing environment is one of the major factors influencing cell growth and survival of the microorganisms, including yeasts. Yeast responds to the nutritional sources present in the surrounding media and also their growth is regulated through the emission of metabolites, toxins and other molecules.

We created the experimental system which allows us to analyze influence of the members of Ras/PKA pathway on the effectiveness of yeast metabolism, cell viability, and selection of the cell death program in naturally during cell growth acidified environment as well as under acid stress conditions.

During the growth of all cells acidification of the medium can be observed. There were no significant medium pH changes in YPD medium. There is possibility that peptone has some buffering effect.

Cell growth rate and amount of accumulated biomass represents replication potential of the yeast, therefore the metabolic activity is partially represented by the ability to change extracellular pH. It is supposed that the ability of yeast cells to grow and survive in particular environment is limited by two main factors – concentration of glucose and pH of the medium (Burtner et al., 2009). Yeast strains are usually grown in the medium containing 2% of glucose. It is known, that decrease of glucose concentration extends replicative cell lifespan (Kaeberlein et al., 2005a; Lin et al., 2000; Kaeberlein at al., 2007).

Second factor that regulates yeast cell growth is the pH of the media. Medium acidification is generally related to the accumulation of acetic acid (Ludovico et al., 2001). Other organic acids are also accumulated in the surrounding environment and they enhance the toxicity of the acetic acid by lowering medium pH (Burtner et al., 2009). Extracellular acidification leads to the intracellular acidification, ant this activates Ras/PKA signal transduction pathway.

We showed that growth rate and the amount of accumulated biomass during the growth of the strains with altered Ras/PKA activity in the medium with the constant pH is several folds higher as compared to the medium with the gradually changing pH. These results proved, that Ras/PKA pathway plays a key role in cell adaptation to the gradually acidified environment. Downregulation as well as hyperactivation of the

pathway disturbs adequate reaction of cell to the changed surrounding, and this led to prolongation or shortening of the cell lifespan.

These results were also confirmed by the Burtner and coauthors (2009). They determined that medium pH and Ras/PKA signal transduction pathway change cell replicative capacity.

Alterations in the activity of Ras/PKA pathway affected yeast cell viability. We showed that members of the Ras/PKA had different functions in relation to the cell viability. Deletion of the *RAS2* gene caused extreme increase in cell viability; therefore deletion of the *RAS1* did not have such effect. It is postulated that main adenylatcyclase controlling gene is *RAS2*; *RAS1* has a secondary function (Sun et al., 1994). Deletion of the both genes causes stopping of the cell cycle in G1 and is lethal to the cell (Toda et al., 1985).

Hyperactivation of the pathway leads to the loss of cell viability of $Ras2^{Val19}$ cells even in early stationary stage in SC medium. Cells of $Ras2^{Val19}$ are insensitive to the changes in their environment and are unable to generate adequate cell response to the stress conditions – they fail to sporulate when starving, fail to accumulate storage carbohydrates when reaching stationary stage (Toda et al., 1987).
Similar results were obtained then analyzing the cell viability of the *ΔPDE2* strain. At the molecular level, both strains have increased amount of cAMP (Hlavata et al., 2003; Thevelein et al., 1999). It was shown, that in the double mutant $Ras2^{Val19}pde1\Delta pde2\Delta$ strain level of cAMP is tenfold higher as compared to wild type strain (Nikawa et al., 1987).

In naturally acidified environment two yeast phosphodiesterases have different roles on the viability of yeast cells. Inactivation of Pde1p caused increase and inactivation of Pde2p caused decrease of the cell viability as compared to SP1.
It is known that Pde2p controls the basic level of cAMP in stationary stage (Park et al., 2005) and protects the cell from extracellular cAMP. Inactivation of this gene causes the loss of homeostasis and leads to the cell death. Meanwhile Pde1p is required for the degradation of the high concentrations of cAMP (Wilson et al., 1993). cAMP concentration is increased in acidic stress conditions.

In all the cases the most medium acidifying yeast strains also had highest cell viability. This show, that cells with high metabolic activity also have protection from the

low pH mechanisms and are able to retain cell viability and prolong cell lifespan. It is postulated, that acetic acid is toxic to the cells that are unable to stop their cell cycle in G1 (Burhans, Weinberger, 2009). It was shown, that accumulation of acetic acid inhibits the arrest of the cell cycle in the stationary stage yeast cultures. Activity of the Ras/PKA is directly related to the arrest of the cell cycle and resistance of the cells during gradual acidification of the medium.

Mutation in the Ras/PKA pathway changes cell response to the stress conditions. *RAS2* is the main regulator acting in yeast cells under stress conditions (Jazwinski, 2002). It is known, that *RAS2* suppresses transcription of the genes containing STRE boxes in their promoters. Constitutive activation of Ras/PKA leads to uncontrolled proliferation and sensitivity to various stress conditions (Broach, 1991). It is known that deletion of *RAS2* causes constant expression of the heat shock protein and increases general stress resistance (Estruch, 2000).

We showed, that inactivation of the *PDE2* also led to loss of cell viability under acid stress conditions. This confirms assumption that increased concentration of cAMP is related to stress sensitivity caused by uncontrolled PKA phosphorylation (Stanhill et al., 1999). Sensitivity to stress correlates with the basic level of cAMP, which is dependent, in stationary stage, on the activity of Pde2p (Park et al., 2005). Additional copy of Pde2p lowers the level of cAMP and causes increased stress resistance.

Testing of the colony formation ability showed that the number of the cells able to produce colonies is lower than the number of the living cells detected during microscopy analysis. Therefore yeast strains with the highest cell viability have also produced highest number of colonies and vice versa. This indicates that resistance to the stress conditions correlates with the replication potential of the cell. Not all the cells are able to replicate after the stress induction.

It is supposed, that Ras/PKA regulates stress response through the general stress response system; by changing localization of the *Msn2* and *Msn4* in the yeast cells (Smith et al., 1998). Increased Ras/PKA activity leads to the phosphorylation of *Msn2* and *Msn4* and this makes them unable to get into nucleus and induce transcription of the stress response genes (Gonze et al., 2008).

Our results show that members of the Ras/PKA pathway that are related to cAMP level in the cell participate in the regulation of the cell viability and aging in gradual acidification and also acidic stress conditions.

Finally, last step of the cell cycle is cell death. Are members of the Ras/PKA related to the regulation of this step of cell life?

There are no discussions about presence of apoptosis in yeast. But the altruistic suicide program is effective only when the cells know they are in population. It is still the question how they sense presence of the neighbors. Gradual acidification caused by accumulation of acetic acid, can be related to the mechanisms of quorum sensing (Fabrizio et al., 2004). It was shown, that combination of the low pH and acetate kills yeast cells with the markers of apoptosis (Ludovico et al., 2001). Ras/PKA pathway regulates many apoptosis related features of the cell: it regulates apoptosis related to actin reorganization (Ho, Bretcher, 2001; Hubbertsey, Mottillo, 2002), increased activity of the pathway is related to accumulation of ROS and cellular respiration (Hlavata et al., 2003).

We showed, that Ras/PKA pathway regulates selection of cell death program of yeast in acidic environment. For this purpose analysis of the early apoptotic marker in analyzed strains was performed.

Nucleus morphology analysis showed that LESS than 5 % of cells with damaged nucleus were registered in YPD and YPD-MES medium. After 78 h of growth in SC medium apoptosis of cells can be observed. Therefore buffering of the medium aborted the degradation of the cell nucleus.

Analysis of the phosphatidylserine exposure, DNA fragmentation and active caspase detection showed similar results. Hyperactivation of the Ras/PKA pathway caused an extreme increase in apoptosis in acidic conditions. Hyperactivation of the Ras/PKA induces programmed cell death also in other fungi, and also in pathogenic like *Candidae albicans* (Philips et al., 2006). Previously it was shown, that in *Ras2*^{Val19} cells the accumulation of the ROS (Hlavata et al., 2003) and increased replicative aging can be observed (Laun et al., 2001; Jazwinski et al.; 1999b, Lin et al., 2000; Steinkraus et al., 2008). Isolation of the mitochondrion of *Ras2*^{Val19} cells showed increased number of proteins with oxidative damage. Problems of *Ras2*^{Val19} dealing with oxidative stress can possibly be related to two main problems: a) constitutively active Ras/PKA, which

effectively stops transcription of the STRE related genes (Hasan et al., 2002); b) PKA independent pathway, which locks respiration in nonphosphorilated mode (Hlavata et al., 2003). Analysis of the lifespan showed, that the increased expression of *PDE2* in *Ras2*^{Val19} also increased the lifespan of the cells.

It is known, that resistance to stress correlates with the basic level of cAMP which is depended on the activity of phosphodiesterase 2 (Russell et al., 1993). Previously was postulated, that an additional copy of *PDE2* can protect the cell with inactive PKA from actin regulated apoptosis. During our research it was shown, that additional copy of *PDE2* plays the same role in acid stress induced apoptosis.

Gourlay and Ayscough (2005) showed that apoptosis can be prevented by increasing *PDE2* expression or deleting of *RAS2* gene. Deletion of the *RAS2* gene causes prolongation of the lifespan of yeast cells by increasing resistance to acetic acid (Burtner et al., 2009). Toxicity of acetic acid is dependent on the concentration. Low doses of the acetic acid induce cell death accompanied by DNA strand breaks, chromatin condensation and externalization of phosphatidylserine, whereas higher doses appear to induce necrosis (Ludovico et al., 2001). Analysis of the acetic acid toxicity showed that in alkaline pH anions of acetate cannot penetrate cell membrane. In acidic conditions acetic acid is found in protonated form and can easily penetrate membrane and induces intracellular acidification (Mollapour et al., 2006). This can explain our result, that buffering of the medium helps prevent cell death. Our results were also confirmed by Burter and coworkers (2009). They showed that buffering of the medium increases the resistance of the stationary stage yeasts by arresting the cell cycle in G1.

Systemic analysis of the influence of the members of Ras/PKA pathway to the yeast metabolism, aging and cell death allowed us to combine this signal system with the regulation of the mentioned processes. It was shown, that the ability to regulate cell functions during gradual acidification and acid stress conditions was related to the activity of the members of Ras/PKA signal transduction pathway. *RAS* genes in these conditions are acting as negative regulators of the cell viability and their activation leads to decreased metabolic activity and induction of apoptosis. Activity of the phosphodiesterase2, conversely, positively regulates the metabolic processes and aborts apoptosis.

Summary of the Ras/PKA regulated targets is presented in figure 5.

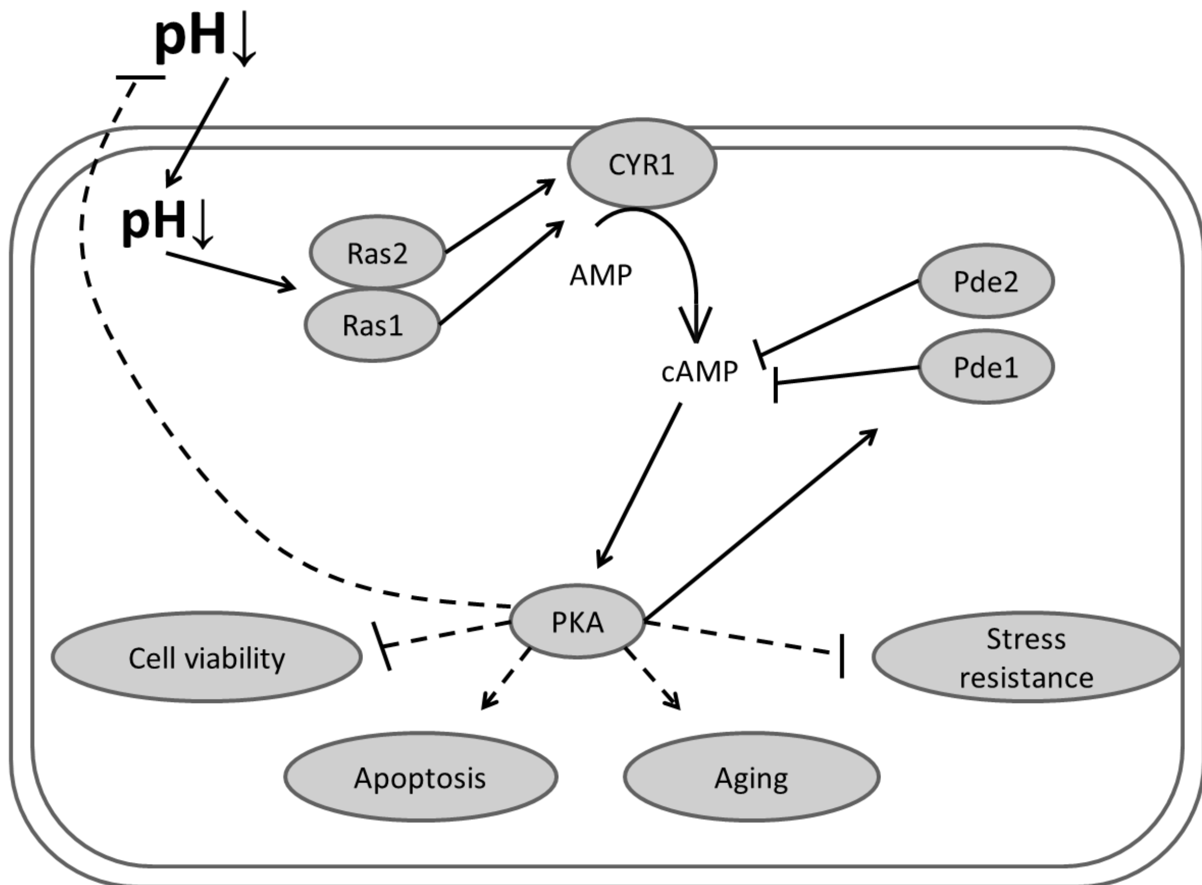

Figure 5. Model of the cell processes controlled by the Ras/PKA signal transduction pathway. *RAS* genes activate *CYR1* and induce cAMP synthesis. Two yeast phosphodiesterases hydrolyze cAMP. Depending on cAMP amount in the cell Pde1p or Pde2p is active. Pde1p is activated by the PKA performed phosphorylation. Activity of PKA induces cell aging and apoptosis, and decreases acid stress resistance, cell viability and metabolic activity in acidic environment. ↑ positive regulation, ┬ negative regulation. Black line – known effect previuosly described in the literature, dotted line – regulation determined in our research.

The pH of the growth medium as well as the activity of the members of Ras/PKA is related to the regulation of chronological and replicative lifespan and selection of the death program in acidic environment. Members of Ras/PKA pathway related to cAMP amount in the cell are directly related to the control of these processes. Our research extend field of appliance of yeast as a model organisms in studies of the mammalian cell aging and death. Conservativity of Ras/PKA is illustrated by the ability to replace yeast genes with appropriate mammalian genes. As a motivation for studying aging processes in yeast was the introduction of oncogenic v-Ha-ras to the yeast cells (Steinkraus et al.,

2008). Analysis of the pH influence on the cell survival can also help understand the proceedings and treatment abilities of the acidosis related disease.

Yeasts are valuable not only as model organisms for studying human processes. Many years they are used in food and alcohol industry. In wine and beer production medium acidification is highly welcome or unwanted. The main component contributing to the quality of wines is concentration of acetic acid. Low concentrations are usually corrected by addition of organic acids (Kapsopoulou et al., 2007). But in production of the best quality wines this procedure is insufficient. Besides particular pH of the medium, also autolysis of the cells is necessary to improve quality of wine. During the yeast cell autolysis peptides and amino acids are released to the medium and they contribute to the production of the highest quality wine (Tabera et al., 2006). Conversely in beer production, too intensive medium acidification is unwanted. Thus ability to control medium acidification and apoptosis is highly desirable in alcohol industry.

CONCLUSIONS

System analysis of influence of the members of Ras/PKA signal transduction pathway on the yeast cell aging and death in naturally acidified environment and under acid stress conditions was performed:

1. *RAS1* and *RAS2* genes act as negative regulators of cell viability during the natural gradual acidification of the medium.
2. Hyperactivation of the Ras/PKA signal transduction pathways activity caused by *Ras2*^{Val19} mutation induces apoptosis in yeast cells (apoptotic markers were detected in 94.25 ± 1.84 %, and this is statistically significant increase as compared to the wild type strain).
3. Downregulation of the Ras/PKA activity caused by deletion of *RAS1* and *RAS2* genes stimulates necrotic death in yeast cells.
4. Activity of the phosphodiesterase2 is related to the termination of the apoptosis in yeast cells: apoptotic death type is detected in cells with inactive Pde2p.
5. Inactivation of the both phosphodiesterase genes induces necrosis in yeast cells.

PUBLICATIONS

1. **Lastauskienė E**., Čitavičius D. Influence of *RAS* genes on yeast *Saccharomyces cerevisiae* cell viability in acid environment, Biologija 2008; 54, 3, 150-155.

2. **Lastauskienė E**., Čitavičius D. *Saccharomyces cerevisiae PDE* genes influence medium acidification and cell viability, Biologija 2009; 55, 1, 24–28.

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SANTRAUKA

Nuolat kintanti aplinka yra pagrindinis veiksnys, kontroliuojantis mikroorganizmų augimą ir vystymąsi. Evoliucijos eigoje organizmuose išsivystė signalinės sistemos, gebančios sujungti aplinkos signalus su ląstelės transkripcijos, transliacijos ir kt. procesais. Viena iš tokių universalių signalinių sistemų yra Ras/PKA signalinis kelias. Ši sistema leidžia mielių ląstelėms reaguoti į aplinkoje esančius maisto medžiagų šaltinius ir įvairius stresinius veiksnius.

Vienas iš pagrindinių aplinkos signalų, įtakojančių ląstelių fiziologiją, yra aplinkos pH. Mielių ląstelėse į aplinkos pH reaguoja Rim101 signalinė sistema, kuri kartu su Ras/PKA reguliuoja tokius procesus kaip sporuliacija ir pseudohifinis augimas. Ekstraląstelinės terpės rūgštėjimas lemia viduląstelinį rūgštėjimą, o tai sukelia mielių ląstelėms stresą ir taip aktyvina Ras/PKA signalinį kelią. Streso poveikio pasekmė yra ląstelių augimo sulėtėjimas, gyvenimo trukmės trumpėjimas, augimo sustabdymas ar netgi žūtis. Pastaraisiais metais parodyta, kad būtent Ras/PKA signalinis kelias reguliuoja ląstelės senėjimo procesą kaip atsaką į aplinkos pH. Taigi mielių, kaip modelinio organizmo, panaudojimas žmogaus genų, reguliuojančių ląstelės senėjimą ir žūtį, tyrimuose vis plečiasi.

Disertacinio darbo metu buvo įvertintas izogeninių mielių kamienų turinčių Ras/PKA signalinio kelio komponentų mutacijas augimas bei mitybinės terpės pH kitimas. Nustatyta, kad mutacijos Ras/PKA signalinio kelio komponentuose lemia skirtingą metabolinį kamienų ląstelių aktyvumą.

Įrodyta, kad Ras/PKA signalinio kelio komponentai įtakoja ląstelių gyvybingumą bei amžių natūralaus laipsniško terpės rūgštėjimo ir rūgštinio streso metu. *RAS* genai šiame procese neigiamai veikia mielių ląstelių gyvybingumą. Neigiama įtaka gyvybingumui rūgštinėmis sąlygomis pasižymi ir *PDE1* genas. Nustatyta, kad pastovaus mitybinės aplinkos pH palaikymas ženkliai pratęsia mielių ląstelių amžių.

Ląstelių žūties būdo analizė parodė, kad padidintas Ras/PKA signalinio kelio aktyvumas dėl *Ras2*^{Val19} mutacijos, laipsniško aplinkos rūgštėjimo ir rūgštinio streso metu, sukelia apoptozę mielių ląstelėse. Kelio aktyvumo sumažėjimas sukeltas *RAS1* ir *RAS2* genų delecijos, priešingai, veikia kaip nekrozės induktorius. Apoptozės procesą stabdo ir fosfodiesterazės 2 aktyvumas mielių ląstelėse. Inaktyvinus abi mielių

fosfodiesterazės mielių kamienuose vyrauja nekrozės procesas. Esant nekintančiam aplinkos pH mažėja ląstelių mirtingumas su apoptozei būdingais žymenimis.

Įrodyta, kad Ras/PKA signalinio kelio komponentai kontroliuoja mielių ląstelių metabolizmo, senėjimo, gyvybingumo ir žūties procesus. Darbo rezultatų pritaikymas praktikoje išplėstų galimybę modeliuoti žinduoliuose vykstančius procesus ir tirti jų reguliaciją mielių ląstelėse. Taip pat kamienai, turintys Ras/PKA signalinio kelio komponentų mutacijas, galėtų būti panaudoti aplinkos rūgštinimo ir apoptozės/nekrozės indukcijos procesuose maisto pramonėje, kur alkoholio bei maisto produktų gamyboje naudojamos mielės *Saccharomyces cerevisiae.*

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Publikacijos:

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Slapšytė G., **Lastauskienė E.,** Mierauskienė J. Genotoxicity of airborne hydrophobic pollutants sampled by semipermeable membrane devices (SPMDs) in Vilnius city, Biologija 2006, 1, 41-46.