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Influence of *RAS* genes on yeast *Saccharomyces cerevisiae* cell viability in acid environment

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During the yeast growth in SD medium (minimal medium with glucose), acidification of this medium can be observed. We determined that two *RAS* genes participate in both extracellular acidification and response to acid environment. In comparison with the wild-type SP1 strain, presence of the *RAS2* gene deletion increases cell viability during gradual medium acidification as well as during acid stress induction. Increased cell viability causes a higher Δ*Ras2* strain growth rate and a higher medium acidification. The medium acidification rate of the strain containing deletion of *RAS1* gene does not differ from SP1. However, cell viability during gradual acidification and in acid stress conditions is significantly higher as compared to SP1. These results suggest that *RAS1* and *RAS2* genes are negative regulators of cell viability in an acid environment, but their contribution to this regulation differs.

Key words: *RAS* genes, medium acidification, cell viability, acid stress

INTRODUCTION

The constantly changing environment is the major factor controlling the growth and development of microorganisms. For many microorganisms, including yeasts, glucose is the preferred carbon and energy source. Glucose affects many important traits of the yeast: growth rate, fermentation capacity and stress resistance. More specifically, yeast cells have developed mechanisms to respond to extreme variations in nutrient availability by modulating their growth and metabolism [1, 2].

Extracellular pH is one of the environmental signals influencing the growth, physiology and differentiation of yeast. During yeast cells growth on a glucose medium, acidification of the medium can be observed [3]. Yeast cells grow more rapidly in a slightly acid medium than in neutral or alkaline media [4]. The extracellular acidity is assumed to arise naturally from respiration / fermentation, CO_2 diffusion and by production of organic acid [5]. Inside the cell, acetic acid dissociates, and if the extracellular pH is lower than the intracellular pH, this will lead to intracellular acidification, anion accumulation and an inhibition of the metabolic cell activity, namely fermentation / respiration [6, 7]. However, this makes only a small contribution to total acidity resulting from glucose-fed yeast metabolism, most of it being due to the action of the plasma membrane H^+-ATPase, with a small contribution from organic acids, predominantly acetate [8]. In *Saccharomyces cerevisiae*, pH response is determined by the RIM101 pathway [9]. Proteolytically processed Rim101p is required for the alkaline pH response. At an acidic pH, Rim101p is inactive in a full length form. The RIM101 pathway participates in the general ion homeostasis of the cell [4, 10, 9].

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RAS proteins are the key regulators of the Ras/PKA pathway which is involved in sensing the nutritional status of a cell. The active GTP-bound form of *RAS* achieves this by regulating the activity of the adenylate cyclase Cyr1p [2]. The polypeptides encoded in the two *RAS* genes are >70% identical overall and approximately 90% identical over the N-terminal residues. The loss of both genes causes an arrest in the G1 phase of the cell cycle [1].

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 [12, 13]. Acidification of the environment through such metabolite as acetic acid, emitted naturally during cell growth, causes the programmed cell death in yeast population [5]. This is the yeast response to a sudden extracellular acidification, whereas the yeast growth under extracellular acidification is slow in a glucose medium and the response of cells to such a long-lasting self-acidification is not definite.

In the present study, we have shown that acidification of the medium causes changes in cell homeostasis, which can induce cell death. Both processes – medium acidification and cell death – are dependent on the activity of the Ras/PKA signal transduction pathway regulating *RAS* genes. It was shown that absence of *RAS1* and *RAS2* genes influence medium pH changes, response to the acidic stress and cell viability.

MATERIALS AND METHODS

Yeast strains, plasmids, growth medium and cultivation conditions

The yeast *Saccharomyces cerevisiae* strains used in the study are listed in Table. Strain SP1 is a wild-type for the genes that regulate activity of the Ras/PKA signal transduction pathway [14, 15]. Other stains used in this research are isogenic to SP1

Table. ***Saccharomyces cerevisiae*** **strains**

Strain	Genotype	Short name	Source of reference
SP1	MATα *his3 leu2 ura3 trp1 ade8 can^R*	**SP1**	20
TK161R2V	MATα *his3 leu2 ura3 trp1 ade8 can^R Ras2^{Val19}*	***Ras2^{Val19}***	20
SP1 *ras1Δras2Δ/p^{21}*	MATα *his3 leu2 ura3 trp1 ade8 can^R ras2::LEU2, ras1::URA3* (*pHa-ras, TRP1*)	***ΔRas1/2***	20
SP1 *ras1Δ*	MATα *his3 leu2 ura3 trp1 ade8 can^R ras1:: URA3*	***ΔRas1***	20
SP1 *ras2Δ*	MATα *his3 leu2 ura3 trp1 ade8 can^R ras2::LEU2*	***ΔRas2***	20
Tr1	MATα *his3 leu2 ura3 trp1 ade8 can^R ras1:: URA3 [pJR1859]*	***Tr1***	This work
Tr2	MATα *his3 leu2 ura3 trp1 ade8 can^R ras2::LEU2 [pJR1244]*	***Tr2***	This work
Tr3	MATα *his3 leu2 ura3 trp1 ade8 can^R Ras2^{Val19}* [*pJR1052*]	***Tr3***	This work

and have mutations in the *RAS* genes, namely *RAS1* and *RAS2* [16]. Deletion of the *RAS1, RAS2* genes in a corresponding yeast increases its resistance to stress conditions. The Δ*Ras1/2* strain contains deletions of both *RAS* genes, and the plasmid presented the human Ha- RAS gene [16] which is postulated to have a structural and functional homology to the yeast *RAS2* gene [17, 7]. The *Ras2^{Val19}* mutation leads to a hyperactivation of this pathway and increases the sensibility to stress conditions [14, 15]. All the strains were kindly provided by Prof. D. Engelberg.

For the yeast transformation, low-copy number plasmids constructed on the base of a 2 μ yeast plasmid were used. For the *Ras2^{Val19}* strain transformation, the pJR1052 plasmid derived by using the pRS314 vector and containing the yeast *RAS2* gene was used (*Tr3*). Δ*Ras1* strain transformation was performed using the pJR1059 plasmid containing the yeast *RAS1* gene and constructed on the base of the YEpLac112 vector (*Tr1*). For the transformation of Δ*Ras2* strain, we used the pJR1244 plasmid containing the yeast *RAS2* gene and constructed by using the pRS424 vector (*Tr2*). All plasmids were kindly provided by Prof. J. Rine [18]. In the following experiments, each of the transformants (*Tr1, Tr2, Tr3*) demonstrate the average of the growth of three independent transformants. The phenotypic characteristics (growth, medium acidification, cell viability) of each independent transformant did not differ statistically significantly.

Yeast transformation was carried out by electroporation according to (http://www.uhmc.sunysb.edu/bioscience/default.htm) or Gietz et al. transformation protocol [19].

Two types of the medium were used for yeast cultivation. Yeasts were grown in a YPD medium containing 2% of glucose, 2% of peptone, 1% of yeast extract, and in an SD medium containing 6.7g/l of yeast nitrogen base (w/o amino acids, with ammonium sulfate), 2% of glucose and supplemented with the appropriate amino acids. When the acidification had to be eliminated, the growth medium was buffered using 2-morpholinoethanesulfonic acid (MES). The initial pH of the media in all cases was 6.2 for YPD and 5.4 for SD media. Yeast cells were grown till the late stationary phase for 78 hours, in an orbital shaker at 130 rpm, 30 °C.

For acid stress induction, sorbiltol 1 M solution was titrated to pH 5.4 and pH 2.1 using HCl and NaOH.

Cell viability evaluation

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

For the microscopy analysis, yeast cells were harvested by centrifugation for 5 min at 5000 rpm, three times washed with PBS (polyphosphate buffer), stained with 50 μM propidium iodide (PI), applied to the microscopy slides and immediately analysed by fluorescent microscopy, 560 nm wavelength (Olympus Provis AX70TRF microscope). For the flow cytometry analysis, 0.5 ml 50 mM Na citrate containing 8 μl/ml PI was added to the harvested yeast cells. The cells were processed immediately. For each sample 20000 cells were counted.

For evaluating the colony formation abilities, yeast cells were grown in liquid media; after 72 hours of growth, the cell number was counted using a Bürker chamber. 100 μl of a known amount of the cells was plated on the YPD medium. The formed colonies were counted after three days of incubation at 30 °C.

For acid stress induction, yeast cells were grown in the YPD medium for 26 hours till the exponential stage, three times washed with PBS buffer and placed in two solutions – pH 5.4 and pH 2.1 – for 4 and 6 hours. After incubation, the viability of the cells was evaluated using microscopy, flow-cytometry or colony formation ability test (as described above).

RESULTS AND DISCUSSION

In this study, we have attempted to determine: a) the influence of *RAS* genes on the acidification of the media during strain growth, b) a relationship between metabolisms caused a gradual acidification of the environment and the viability of yeast cells. For the analysis of these processes, yeast cells were grown for 78 hours in YPD and SD media; the pH of the media and the viability of cells were measured each 6 hours.

During yeast cell growth in a YPD medium, independently of the genotype of the strain, only a slight acidification of the medium was observed: from the starting pH 6.2 to pH 4.9 after 72 hours of growth. The medium acidification, as compared among the strains used, was statistically insignificant ($p > 0.05$). Such stability of the medium pH may be caused by a possible buffering effect of peptone.

A significant medium acidification was observed when yeast strains were grown in an SD medium (Fig. 1, B). According to the ability to change extracellular pH, yeast strains were divided into two groups. Statistically significant differences of the medium acidification between these two groups could be seen already after 12 hours of growth.

The first group slightly acidified the growth medium (pH 5.4 ± 0.00–3.15 ± 0.07). This group contained SP1, *Ras2*^{Val19}, Δ*Ras1* strains and *Tr1* and *Tr3* transformants.

The second group of strains was determined by a significant acidification of the medium (pH 5.4 ± 0.00–2.04 ± 0.05). This group comprised Δ*Ras1/2 (*supposed to have a wild-type genotype in relation to the *RAS2* gene), Δ*Ras2* strain and *Tr2* transformants. The level of the medium acidification significantly differed from the first group of strains.

A comparison of growth in SD medium and medium acidification has shown that the ability to acidify the medium directly correlates with the growth of yeast (Fig. 1. A, B). It should be mentioned that in YPD medium the growth rate showed no statistically significant differences between the strains (data not shown).

To explain such results, we have hypothesized that *RAS2* deletion reduces strain sensitivity to stress in an acid medium. This causes a steady cell viability which determines a better growth and consequently a higher medium acidification rate. The proposed hypothesis predicts that the yeast *RAS2* gene in an acid environment acts as a negative regulator of cell viability, and deletion of *RAS2* blocks the following effect.

Our results confirm this assumption: all strains of the first group (Fig. 1, B) were more sensitive to pH changes, therefore showed a poor growth.

Analysis of the growth and acidification rate of Δ*Ras1/2* strain and *Tr2* transformants has shown that the human HaRAS gene and the yeast *RAS2* gene, when placed in low-copy number plasmids, are unable to fully suppress *RAS2* deletion. In both cases, cell growth and medium acidification rates were not restored to the SP1 level. The stability of plasmids in all transformed strains was 65.87 ± 3.45%. These results show that *RAS2* deletion causes extreme changes in the metabolic activity of the cell. In *Tr2* transformants, a low-copy number plasmid is not sufficient to ensure the wild-type level of medium acidification.

To confirm the assumption of the negative effect of the *RAS2* gene on cell viability in an acid environment, we performed cell viability analysis in a long-lasting gradual medium acidification and under acid stress conditions.

It is known that acidification of the media is related with cell death. Yeast cells are committing the programmed cell death in response to acetic acid which is a normal end product of the alcoholic fermentation carried out by *Saccharomyces cerevisiae* [5].

Microscopy data on cell viability showed that during yeast cell growth in YPD and YPD-MES media there were no significant differences between the growth of strains with different *RAS* gene mutations and SP1 strain. The viability of cells varies from 99.43 ± 0.26% to 87.97 ± 4.62% in different strains (data not shown), and these differences were not statistically significant. Cell viability remained to be over 87.00% even in the late stationary phase (72 hours) in all strains independently of their genotype.

Analysis of yeast cell viability after 22 and 46 hours of growth in SD and SD-MES media showed no differences between the strains or between the selected media (data not shown). Data presented in Fig. 2 (part A) show a statistically significant difference in the cell viability of different strains grown in the abovementioned media after 72 hours of growth. The viability of cells of SP1, *Ras2*^{Val19}, *ΔRas1* strains and *Tr1, Tr2, Tr3* transformants was significantly higher in the medium with a constant pH (SD-MES) as compared to the SD medium and to the SP1 strain ($p < 0.005$). Exceptions were in cases of *ΔRas1/2* and *ΔRas2* strains where the viability of the cells in SD or SD-MES media did not differ statistically significantly. *RAS2* gene deletion seems to cause a defect in cell response to the changed environmental pH. In the case of the *RAS1* gene deletion, cell viability was lower in SD medium, but still it was significantly higher as compared to the SP1 strain ($p = 0.0031$). Transformation of the strains with a low-copy number plasmid significantly decreased cell viability as compared to the parent strains ($p < 0.005$), and the Tr1 viability reached the SP1 strain level.

After 72 hours of growth in SD medium, 34.17% of wild-type SP1 cells were alive, and the pH value at this point was 3.3. An increased viability of cells was observed in the case of Δ*Ras1/2* ($p = 0.0001$) with pH 2.31, Δ*Ras1* ($p = 0.0031$) – pH 3.36, Δ*Ras2* ($p = 0.0001$) – pH 2.29; *Tr1* ($p = 0.0456$) – pH 3.15, *Tr2* ($p = 0.0039$) with pH 2.04 as compared to the wild-type cells. Comparison of strains *Ras2*^{Val19} and SP1 showed that the cell viability strongly differs after 72 hours ($p = 0.0153$) and even after 46 hours of growth ($p = 0.0077$). A decreased cell viability was also seen in the case of *Tr3* ($p = 0.0370$) with pH 3.44 in the meas-

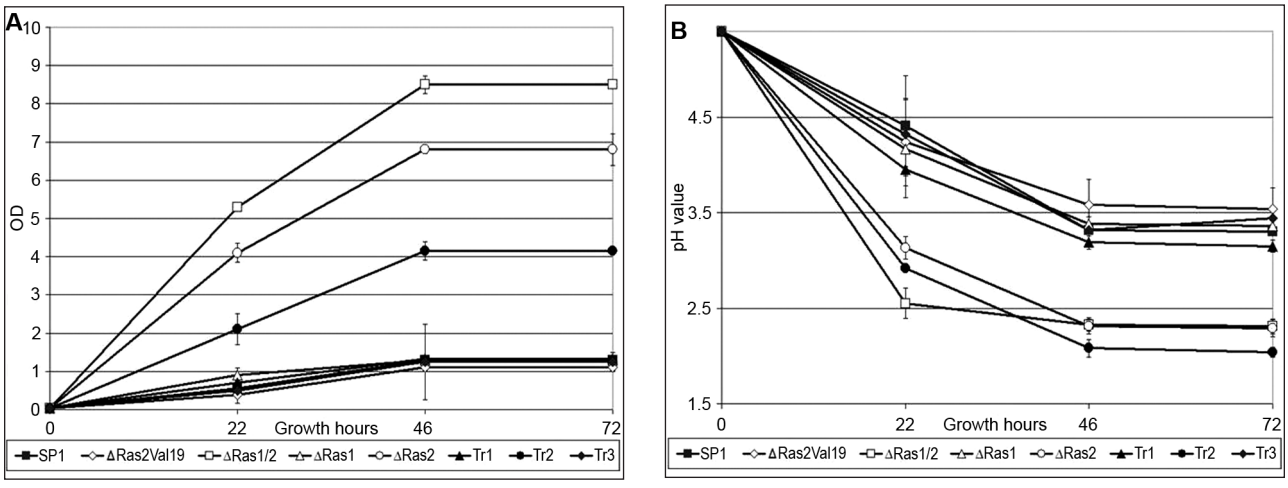

Fig. 1. Yeast strain growth (A) and acidification (B) in liquid SD medium. Error bars indicate standard deviation. Experiments were independently repeated three times

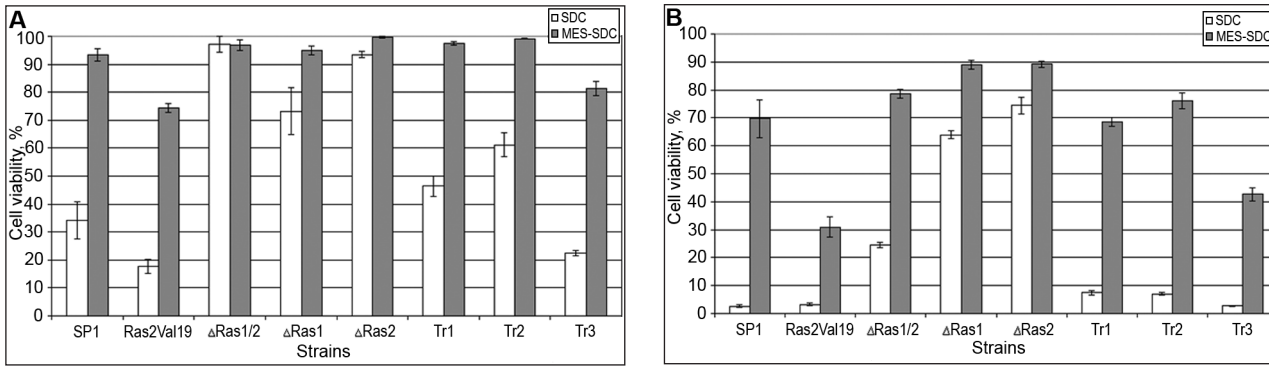

Fig. 2. Cell viability in SD and buffered SD-MES media (pH 5.4). Samples were taken after 72 h of growth and analysed by UV microscopy, >560 nm wavelength (A) or by flow-cytometry (B). Error bars indicate standard deviation. Experiments were independently repeated three times

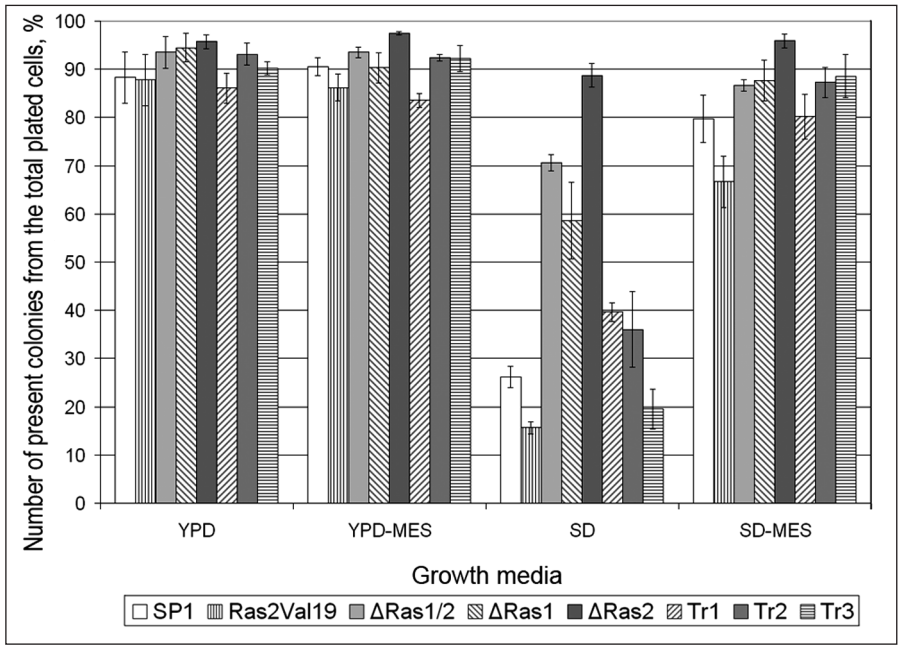

Fig. 3. Colony formation ability after 72 h of cell growth in different media. Yeast cells were grown in liquid media for 72 h, later samples were taken and a known amount of cells was plated on SD plates. Colonies were counted after 48 h. Error bars indicate standard deviation. Experiments were independently repeated three times

ure point as compared to SP1. These results show a positive effect of *RAS* gene deletion on cell viability in an acid environment.

This assumption was supported by flow-cytometry (Fig. 2, B). Differences among the growth in buffered/nonbuffered media of SP1, *Ras2*^{Val19} strains and Tr1, Tr2, Tr3 transformants were wide. The data also showed that deletions of the *RAS1* and *RAS2* genes increased cell viability as compared to SP1 ($p < 0.005$). The discrepancy between the two methods (microscopy and flow-cytometry) could occur because the sensitivity of flow-cytometry is much higher. During cell staining with PI, a different intensity of staining can be observed. In the microscopy analysis, only completely red stained cells are counted as "dead", while in flow-cytometry even slightly stained (light red) cells are registered as "positive". No changes in the Δ*Ras1/2* strain viability were seen in microscopy data; however, flow-cytometry data showed a statistically significant decrease of the viability of the Δ*Ras1/2* grown in SD media. Such differences between the two methods can occur because of a high autofluorescence of Δ*Ras1/2* cells. Regardless of differences between microscopy and flow-cytometry results, the tendency of strain cell viability is the same.

Cell ability to produce a colony-forming unit is visibly the best method to determine cell viability. The data on colony formation abilities are presented in Fig. 3. No differences among the strains were observed as the cells were grown in YPD, YPD-MES and SD-MES media. Colony formation ability test results, after the growth in SD medium, showed the same tendency of cell viability as in microscopy and flow-cytometry: a significant cell viability increase as compared to the SP1 was observed in Δ*Ras1/2,* Δ*Ras2* and Δ*Ras1* strains. The viability of the transformants did not differ from the wild-type SP1 strain ($p > 0.005$).

The cell viability of SP1, Δ*Ras1,* Δ*Ras1/2* strains and *Tr1, Tr2, Tr3* transformants strongly decreased during the growth in SD medium as compared to the growth in SD-MES medium. The Δ*Ras2* strain viability did not change significantly when grown in different media.

According to cell viability in SD medium, yeast strains fall into two groups that remind the previously discussed two groups when the ability to acidify the growth medium was evaluated. Differences were observed in case of Δ*Ras1* stain and *Tr2* transformants. The Δ*Ras1* strain, belonging to group 1 (according to

medium acidification), retains a high viability of the cells, whereas *Tr2* transformants which significantly acidify SD media (group 2) have a low viability of cells. In case of other strains, a direct correlation can be seen: a significant acidification correlates with a high viability (group 2) and a low ability to acidify media leads to a low cell viability (group 1).

These results show a possible participation of *RAS* genes in the regulation of processes involved in growth medium acidification. To confirm this assumption, acid stress induction was carried out.

Acid stress was achieved by placing yeast cells in solutions with pH 5.4 and 2.1 (Fig. 4). The pH of the incubation solution was chosen according to the natural acidification of the media during yeast strain growth (Fig. 1). Various incubation periods were tested (from 1 to 8 hours); the viability differences of the strains were revealed after 4 and 6 hours of incubation.

After incubation in 5.4 pH solution, only the viability of the $Ras2^{Val19}$ strain decreased as compared to SP1 ($p < 0.005$) (Fig. 4, A).

Data on yeast cell survival in the pH 2.1 solution show a decrease in cell viability of SP1 (Fig. 4, A). $Ras2^{Val19}$ strain survival was significantly lower after incubation in the 2.1 pH solution (as compared to the wild-type strain) and decreased during the incubation. The viability of the Δ*Ras1/2* and Δ*Ras1* strains was significantly higher as compared to SP1 ($p < 0.005$). A high level of cell survival under acid stress conditions was noted in the case of Δ*Ras2*: even after 6 hours of incubation in the 2.1 pH solution, over 68% of the cells were viable. The viability of *Tr1*, *Tr2*, *Tr3* transformant strains did not significantly differ from that of SP1. These results were also confirmed by flow-cytometry (Fig. 4, B) and the colony formation ability test (Fig. 4, C). As previously, flow-cytometry data showed a generally lower cell viability than the microscopy results.

Results of acid stress induction have confirmed the assumption that deletion of the *RAS2* gene provides for yeast cells resistance to the acidic stress. Different responses of the *RAS1* and *RAS2* genes to the stress were shown previously in the context of response to heat stress [20]. *RAS2*, along with *RAS1*, is required to increase the life span when yeasts are exposed to a transient, sublethal heat stress. *RAS2* is necessary to downregulate stress response genes and to upregulate genes necessary for growth. However, the requirement for *RAS1* is perplexing because this gene is redundant to *RAS2* [20].

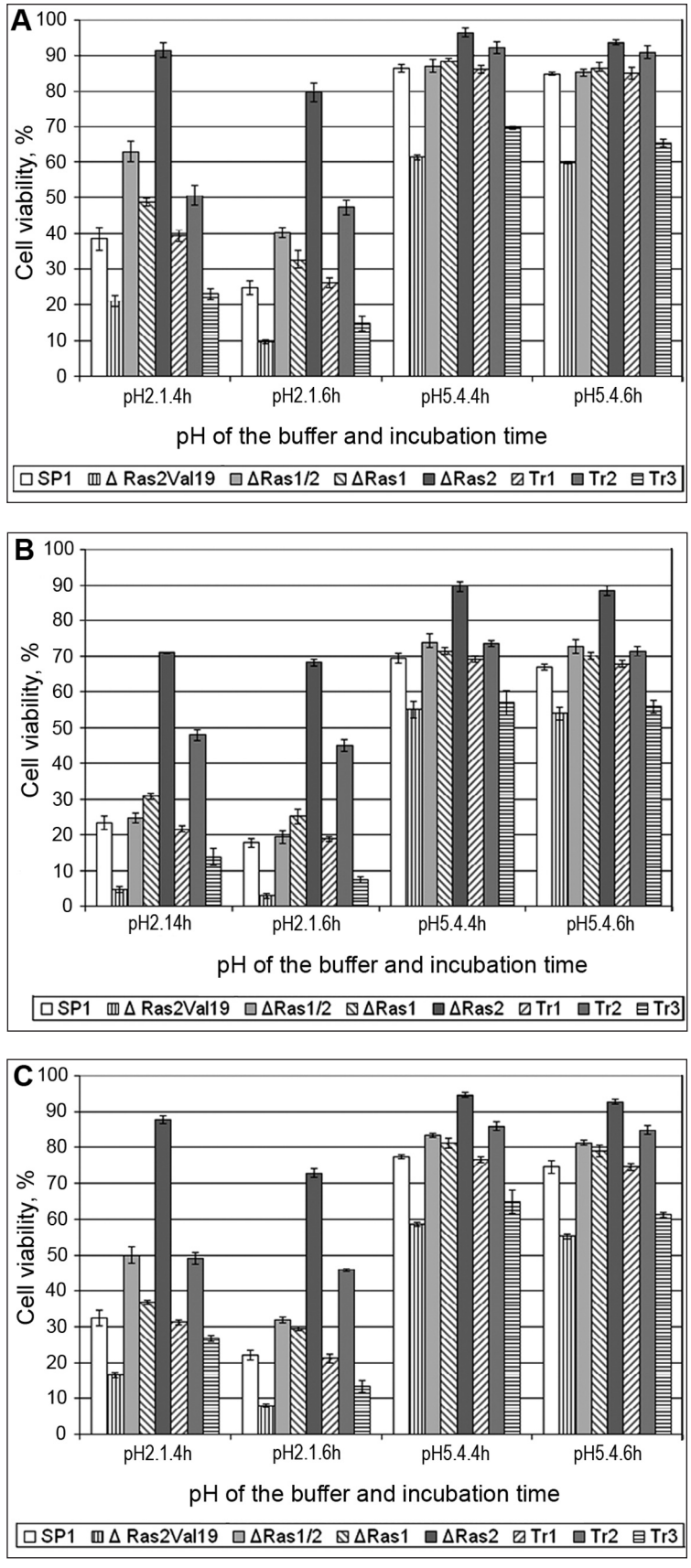

Fig. 4. Acid stress induction. Yeast cells were placed into two solutions (pH 5.4 and pH 2.1) for 4 h and 6 h. After the acid stress induction, cell viability was evaluated by fluorescent microscopy (A), flow-cytometry (B) and colony formation (C). Error bars indicate standard deviation. Experiments were independently repeated three times

Ras2^{Val19} mutation increases the sensitivity of yeast cells to stress conditions [21, 22]. This strain is also sensitive to acid stress.

The Δ*Ras1* strain is more sensitive to acid stress than the Δ*Ras2* strain, but more resistant than SP1. Results of the acid stress show a decrease of cell viability with the incubation time. Analogous results were obtained also for the Δ*ras1*/*2* strain.

Transformant *Tr2* also showed an increased resistance to acid stress. This rise in cell viability can be caused by the population of cells which have lost a plasmid, are non-dividing and viable. On the other hand, this effect can be caused by incomplete suppression of the deletion of *RAS2* gene by the low-copy number plasmid. Deletion of the *RAS2* gene causes low PKA activity and induces genes by multiple stress response [23].

Our data show that absence of *RAS2* gene product increases cell resistance to acid stress conditions and to a prolonged incubation in an acidic medium. The Δ*ras1* strain is more sensitive than Δ*ras2*. In conclusion, these results demonstrate that *RAS* genes, especially the *RAS2* gene, act as negative regulators of the *Saccharomyces cerevisiae* viability under acidic conditions.

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RAS GENŲ ĮTAKA MIELIŲ (*SACCHAROMYCES CEREVISIAE*) LĄSTELIŲ GYVYBINGUMUI RŪGŠTINĖJE APLINKOJE

Santrauka

Mielėms augant SD terpėje (minimali terpė su gliukoze), terpė rūgštėja. Nustatyta, kad *RAS* genai dalyvauja tiek ekstraląsteliniame rūgštėjime, tiek ir atsake į rūgštinę aplinką. Lyginant su laukinio tipo SP1 kamienu, esant *RAS2* geno delecijai, ląstelių gyvybingumas padidėja tiek laipsninio terpės rūgštėjimo, tiek ir rūgštinio streso metu. Padidėjęs ląstelių gyvybingumas lemia didesnį *ΔRas2* kamieno augimo greitį bei didesnį terpės rūgštėjimą. Tuo tarpu *RAS1* geno deleciją turintis kamienas mitybinę terpę rūgštino taip pat kaip ir SP1, tačiau ląstelių gyvybingumas ir laipsninio rūgštėjimo, ir rūgštinio streso sąlygomis buvo statistiškai patikimai didesnis nei SP1. Tai rodo, kad *RAS* genai neigiamai veikia ląstelių gyvybingumą rūgštinėje aplinkoje, tačiau jų indelis šiuo atveju nevienodas.