ISSN 0026-2617, Microbiology, 2007, Vol. 76, No. 2, pp. 158–163. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © E.N. Biryukova, A.G. Medentsev, A.Yu. Arinbasarova, V.K. Akimenko, 2007, published in Mikrobiologiya, 2007, Vol. 76, No. 2, pp. 184–190.

EXPERIMENTAL ARTICLES

Adaptation of the Yeast *Yarrowia lipolytica* to Heat Shock

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Received April 8, 2006

Abstract—The adaptive response of the yeast *Yarrowia lipolytica* to heat shock has been studied. Experiments showed that, after 10 min of incubation at 45°C, the survival rate of *Yarrowia lipolytica* cells was less than 0.1%. Stationary-phase yeast cells were found to be more thermotolerant than exponential-phase cells. A 60-min preincubation of cells at 37°C or pretreatment with low concentrations of H_2O_2 (0.5 mM) or menadione (0.05 mM) made them more tolerant to heat and to oxidative stress (120 mM hydrogen peroxide). The pH dependence of yeast thermotolerance has also been studied. The adaptation of yeast cells to heat shock and oxidative stress was found to be associated with a decrease in the intracellular level of cAMP and an increase in the activity of antioxidant enzymes (catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, and glutathione reductase).

DOI: 10.1134/S0026261707020051

Key words: yeast, *Yarrowia lipolytica*, heat shock, oxidative stress, survival, adaptation, cAMP, catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, glutathione reductase.

Every yeast is characterized by a particular range of temperatures optimal for growth. At higher temperatures, the growth of yeasts slows down or even stops, the survival rate often decreasing [1, 2].

Various stresses, including heat shock, cause global changes in cell metabolism and inflict damage on cellular membranes and proteins. In response, microorganisms can activate adaptive mechanisms which provide for their survival and increase in competitive ability [3].

The adaptive mechanisms of yeasts are as yet poorly understood. The relevant data available in the literature are ambiguous. Further studies along this line could provide better insight into the problem.

The aim of this work was to investigate the dependence of the thermotolerance of the yeast *Yarrowia lipolytica* on various factors that can influence cell survival under stressful conditions. Attempts were also made to study the correlation between cell adaptation and changes in the cellular content of cAMP and antioxidant enzymes.

MATERIALS AND METHODS

The yeast *Yarrowia lipolytica* VKM Y-2378 (= *Y. lipolytica* Y-155) used in this study was obtained from the All-Russia Collection of Microorganisms (VKM). The yeast was cultivated at 29°C on a shaker (200 rpm) in 750-ml flasks with 100 ml of Reader medium containing 1% glucose as the source of carbon and energy [4]. Growth was monitored by measuring culture turbidity at 540 nm. The grown cells were harvested by centrifugation, washed with sterile distilled water, and resuspended in 50 mM phosphate buffer (pH 7.0).

Heat shock was induced by incubation of exponential-phase (10–12 h of growth) or stationary-phase (24 h of growth) cells at 45°C. Mild thermal pretreatment of yeast cells was carried out by incubating them at 37°C for 60 min.

The survival rate of cells was determined by plating them on malt extract agar. The plates were incubated at 29°C for 48–72 h. Then the number of grown colonies was determined. The data presented in this paper are the average results of triplicate experiments.

The effect of pH on yeast thermotolerance was studied as follows: exponential-phase cells were harvested by centrifugation, washed with sterile distilled water, and resuspended in 5 mM phosphate buffer (pH 7.0). The pH of aliquots of this suspension was adjusted to 3.0, 4.5, 6.0, and 7.2. The aliquots with different pH values were preincubated at 29°C for 60 min and then incubated at 45°C. At regular intervals, cells were taken to assay their survival.

cAMP was extracted from yeast cells with 5% perchloric acid. For this purpose, 0.9 ml of cell suspensions incubated under the considered conditions (incubation at 37 and 45°C and incubation in the presence of

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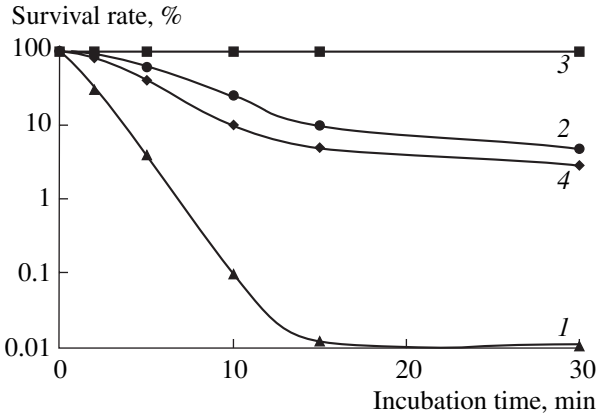

Fig. 1. The survival rate of *Y. lipolytica* cells from the exponential (curves *1, 3, 4*) and the stationary (curve *2*) growth phases at (*1, 2*) 45 and (*3*) 37°C. Curve *4* shows the survival at 45°C of the yeast cells that were preliminarily incubated at 37°C for 60 min.

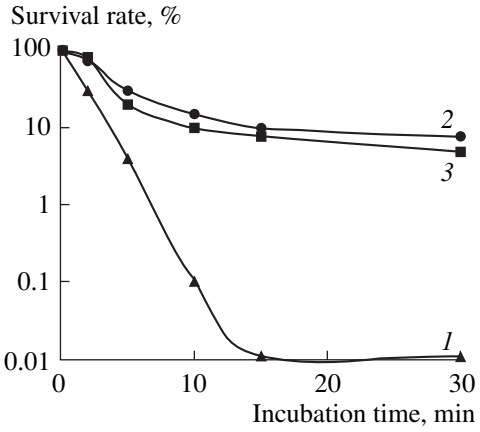

Fig. 2. The survival rate at 45°C of *Y. lipolytica* cells: (*1*) untreated, (*2*) pretreated with 0.5 mM H_2O_2 for 60 min, and (*3*) pretreated with 0.05 mM menadione for 60 min.

H_2O_2) was placed in Eppendorf tubes containing 0.1 ml of 50% perchloric acid and thoroughly mixed. The mixture was incubated in ice bath for 10 min, neutralized by adding 5 N KOH with vigorous mixing, and centrifuged at 10000 *g* for 10 min. The pellet was discarded. The supernatant was stored at –40°C if it could not be used immediately. cAMP was assayed by a routine procedure using a reagent kit from Amersham.

Antioxidant enzymes (catalase, superoxide dismutase, glutathione reductase, and glucose-6-phosphate dehydrogenase) were assayed in a cell-free extract as described earlier [4]. To prepare the extract, cells were washed twice with distilled water and suspended in 50 mM Tris-phosphate buffer (pH 7.0) containing 0.5 mM phenylmethanesulfonyl fluoride (PMSF, an inhibitor of proteases). The suspension was disrupted in a French press. The homogenate was centrifuged at 105000 *g* for 60 min. The pellet was discarded, and the supernatant was used for enzyme assay.

Protein was quantified with the biuret reagent.

Spectral measurements were carried out using a Shimadzu UV-160 spectrophotometer (Japan).

The reagents used were a 3% solution of locally produced H_2O_2; cytochrome *c* purchased from Sigma; and PMSF, menadione, xanthine, and xanthine oxidase purchased from ICN.

RESULTS AND DISCUSSION

Figure 1 illustrates the effect of heat shock on the survival rate of yeast cells taken from different growth phases. As can be seen from this figure, incubation at 45°C diminished cell survival, so that only 4% and less than 0.1% of cells remained viable after 5 and 10 min of incubation, respectively (Fig. 1, curve *1*). Stationary-phase cells were found to be more thermotolerant (after 30 min of incubation at 45°C, 60% of cells remained viable) (Fig. 1, curve *2*).

The study of the effect of mild thermal pretreatment on the thermotolerance of yeast cells showed that their incubation at 37°C for 60 min virtually did not affect cell viability (Fig. 3, curve *3*). At the same time, the above pretreatment increased (up to 40%) the survival rate of yeast cells during 30-min incubation at 45°C (Fig. 1, curve *4*).

Sanchez and Lindquist also showed that the preliminary exposure of *Saccharomyces cerevisiae* cells to 37°C induces their tolerance to the subsequent action of higher temperatures [5]. This effect is known as induced thermotolerance or heat hardiness.

It is known that microorganisms exposed to a particular stress can acquire tolerance to another stress [3]. In

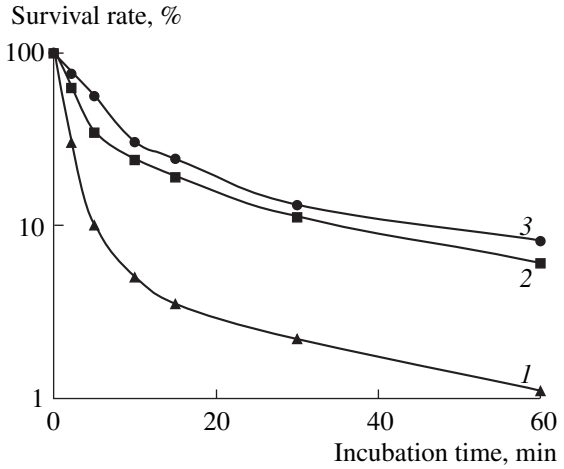

Fig. 3. The effect of thermal pretreatment and pretreatment with a low concentration of oxidant on the survival rate of *Y. lipolytica* cells: (*1*) untreated, (*2*) preincubated at 37°C for 60 min, and (*3*) pretreated with 0.5 mM H_2O_2 for 60 min.

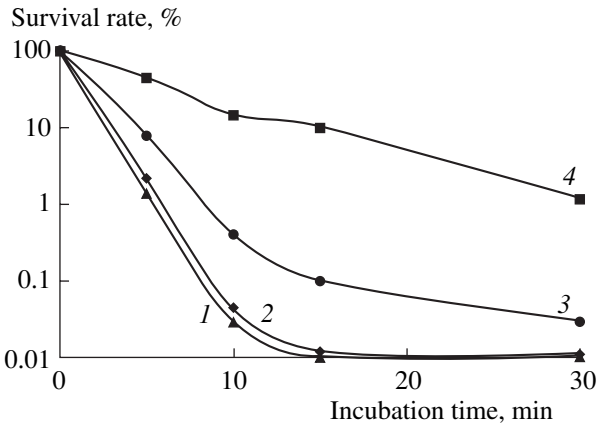

Fig. 4. The effect of thermal pretreatment (29°C, 60 min) at different pH values on the survival rate of exponential-phase *Y. lipolytica* cells at 45°C: (*1*) pH 6.0, (*2*) pH 7.2, (*3*) pH 3.0, (*4*) pH 4.5.

our earlier work [4], we studied how the pretreatment of yeast cells with nonlethal concentrations of oxidants (H_2O_2 or menadione, a superoxide-generating agent) can affect their thermotolerance. The 60-min pretreatment of exponential-phase cells with a nonlethal concentration of H_2O_2 (0.5 mM) enhanced (up to a value of 30%) their survival during subsequent incubation at 45°C (Fig. 2, curve *2*). Similar results were obtained when exponential-phase cells were pretreated with a nonlethal concentration of menadione (0.05 mM) (Fig. 2, curve *3*).

It was also of interest to study the ability of the heat-pretreated yeast to acquire tolerance to oxidative stress. As shown in Fig. 3, the thermal pretreatment of yeast cells enhanced their tolerance to oxidative stress induced by 120 mM H_2O_2 (curve *2*). The effect of thermal pretreatment on cell survival was the same as in the case of pretreatment with 0.5 mM H_2O_2 (curve *3*).

Thus, *Y. lipolytica* cells are capable of developing cross-tolerance to various stresses.

The relevant data available in the literature are scarce and ambiguous. Mutoh et al. [6] and Watson [7] showed that *Schisosaccharomyces pombe* cells treated with a nonlethal concentration of H_2O_2 acquire tolerance to lethal concentrations of H_2O_2, heat shock (48°C), and ethanol stress. On the other hand, the exposure of *S. cerevisiae* cells to H_2O_2 [8] or menadione [9] does not induce their tolerance to the subsequent action of elevated temperatures and, conversely, the exposure of *S. cerevisiae* cells to 37°C does not induce their tolerance to lethal concentrations of H_2O_2 [8]. Studies by electrophoresis have shown that the pretreatment of yeast cells with menadione induces the biosynthesis of only one polypeptide with a molecular mass of 60 kDa [9], whereas incubation at 37°C induces the biosynthesis of as many as ten proteins [8, 9]. These data may indicate that the effect of various stresses on *S. cerevisiae* cells is rather specific. This specificity is probably determined by the fact that different stresses act on different cellular targets.

The pH value of the medium is an important factor that controls many cellular functions. The effect of elevated temperatures on yeast cells is associated with a significant, although slow, decrease in intracellular pH, changes in ATPase activity, and induction of heat-shock proteins [10].

Figure 4 shows the effect of pH value on the survival of *Y. lipolytica* cells exposed to heat shock. The preincubation of these cells at pH 6.0 and 7.2 (Fig. 4, curves *1* and *2*) virtually did not influence their survival rate at high temperatures (Fig. 1, curve *1*). However, the preincubation of yeast cells at pH 3.0 enhanced their survival (Fig. 4, curve *3*). The highest degree of survival was observed when cells were preliminarily incubated at pH 4.5 (Fig. 4, curve *4*). Similar induction of thermotolerance in *S. cerevisiae* cells incubated at low pH values was observed by Coote et al. [11]. The incubation of cells at elevated temperatures (within a range of 37 to 45°C) was found to cause an acidification of the medium and to enhance cell tolerance to lethal temperatures. These effects probably depend on ATPase, since diethylstilbestrol (an inhibitor of ATPase) diminishes both the medium acidification and the enhancement of cell thermotolerance. The preincubation of yeast cells at pH 4.5 enhances their thermotolerance. It can be anticipated that the enhanced thermotolerance of yeast cells is due to their increased ability to maintain a normal value of intracellular pH. Indeed, ^{31}P-NMR studies showed that yeast cells preincubated at a sublethal temperature show a better ability to maintain intracellular pH compared to unadapted cells [11]. These data suggest that pH is directly involved in the functioning of thermotolerance mechanisms in *S. cerevisiae*.

Intracellular pH decreases in response to various stresses, including exposure to heat and ethanol [10–12]. Variations in intracellular pH can influence important cellular functions, such as cAMP metabolism [13]. Exposure of cells to heat and other stressful factors induces their genetic activity associated with secondary messengers [13, 14].

Changes in the direction of genetic transcription activate the synthesis of the mRNAs that encode heat-shock proteins. Such proteins were found in virtually all cellular structures and compartments, including cytoplasm, nuclei, and mitochondria [15]. This suggests that heat-shock proteins are multifunctional agents required for the maintenance of cell viability under heat shock.

The measurement of the cAMP content in *Y. lipolytica* cells exposed to heat showed that, as early as after two minutes of incubation at 45°C, the intracellular concentration of cAMP began to increase, reaching a maximum after four minutes of heat treatment (Fig. 5, curve *2*). During the next two minutes, the content of cAMP in the cells tended to decrease and reached a value of less than 50% of the initial content after 10–

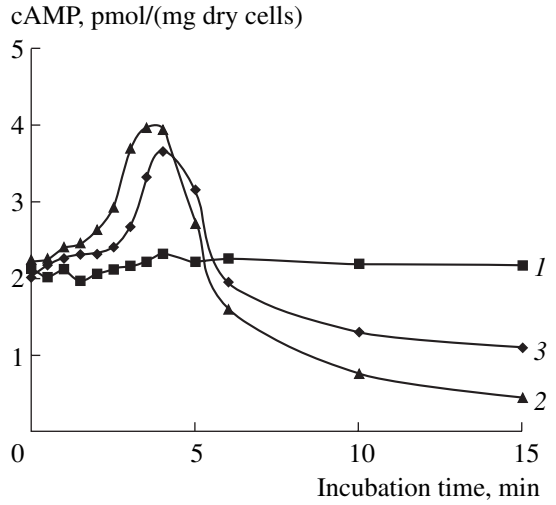

Fig. 5. The content of cAMP in *Y. lipolytica* cells exposed to (*1*) 29, (*2*) 45, and (*3*) 37°C.

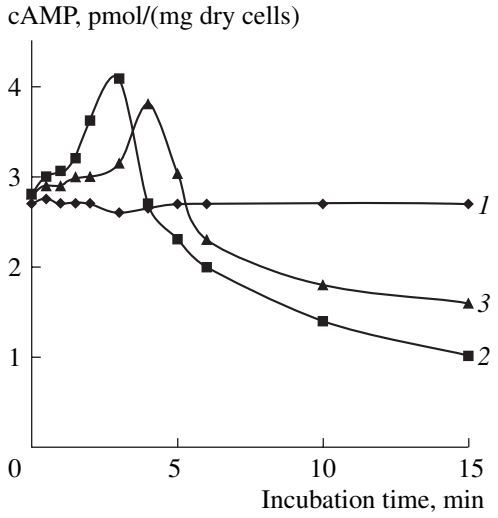

Fig. 6. The content of cAMP in *Y. lipolytica* cells incubated (*1*) without oxidant and in the presence of (*2*) 120 and (*3*) 0.5 mM H_2O_2.

15 min of heat treatment. In the control (yeast cells incubated at the optimal growth temperature of 29°C), the content of cAMP virtually did not change (Fig. 5, curve *1*). In cells exposed to 37°C (the temperature that was used to enhance cell thermotolerance), the cAMP content changed in the same manner (Fig. 5, curve *3*) as in cells incubated at 45°C.

When yeast cells were incubated in the presence of 120 mM H_2O_2 (severe oxidative treatment), the cellular content of cAMP changed as in the case of heat treatment, showing an initial twofold rise (Fig. 6, curve *2*), followed (after three minutes of incubation) by a steep decrease to a value lower than the initial.

When yeast cells were incubated in the presence of 0.5 mM H_2O_2 (mild oxidative treatment), the cellular content of cAMP attained a maximum after four minutes of incubation (Fig. 6, curve *3*) and then tended to decrease, but not so steeply as in the case of the severe oxidative treatment.

A comparison of Figs. 5 and 6 showed that, in response to both heat and oxidative stresses, the cellular content of cAMP increases by more than twofold during the first two–four minutes and then drastically decreases to a level that is lower than the initial cAMP content.

It should be noted that similar results were obtained in our earlier study of the effect of KCN (an inhibitor of respiration) and high concentrations of ethanol and acetate on *Y. lipolytica* cells [16] and were reported by other authors for *Neurospora crassa* cells exposed to heat and some agents diminishing the membrane potential of the cytoplasmic membrane [14, 17].

It is known that the content of cAMP in cells is determined by the ratio of the activities of adenylate cyclase and phosphodiesterase, the enzymes that catalyze the synthesis and breakdown of cAMP, respectively. We may suggest that the increase in the cellular content of cAMP observed during the first minutes of cell exposure to heat (Fig. 5) or H_2O_2 (Fig. 6) is due to increased adenylate cyclase activity. This suggestion is confirmed by the data of Trevelein, who showed that heat shock induces adenylate cyclase activity in yeasts [13]. The subsequent decrease in adenylate cyclase activity and increase in phosphodiesterase activity may be due to a decline in intracellular pH [13, 16].

The effect of cAMP on stress-induced genes involves cAMP-dependent protein A kinase and depends on the presence of cAMP-responsive elements (CREs) in these genes. CRE was first characterized as a regulatory element in the somatostatin gene of rats. Upon the transcriptional regulation of the rat somatostatin gene, an increase in the cAMP level induces phosphorylation of the CRE-binding factor, CREB. In turn, the phosphorylated CREB induces the somatostatin gene. In contrast to the CREs of vertebrates, which act as gene enhancers, the CRE of *Neurospora crassa* acts as a gene switch at high cAMP levels. When the cAMP level decreases, the phosphorylated form of CREB is dephosphorylated, and the CREB dissociates from the CRE, activating the transcription of the gene [18]. The key role of cAMP in the development of yeast tolerance to heat shock is confirmed by experiments on yeast mutants defective in adenylate cyclase. The low level of cAMP in these mutants accounts for their low growth rate at the optimal temperature and high tolerance to heat shock [19]. In contrast, when the cAMP level is high, the thermotolerance of yeast cells decreases [20].

In response to heat shock, the yeast *S. cerevisiae* enhances the production of reactive oxygen species (ROSs), as a result of which the viability of yeast cells

Activity of antioxidant enzymes in *Y. lipolytica* cells exposed to heat shock

Cells	Growth phase	Enzyme activity, μmol/(min mg protein)			
		Catalase	Superoxide dismutase	Glucose-6-phosphate dehydrogenase	Glutathione reductase
Untreated	Exponential	28.0 ± 3.3	4.2 ± 0.45	73.0 ± 2.3	24.0 ± 2.1
Untreated	Stationary	75.0 ± 8.3	8.4 ± 1.1	103.0 ± 4.0	69.0 ± 1.3
Pretreated with heat (37°C, 60 min)	Exponential	105.0 ± 0.5	11.2 ± 2.1	120.0 ± 1.3	40.0 ± 0.7

decreases. The mutant cells defective in antioxidant enzymes are less tolerant to heat than normal cells and, conversely, the mutant cells with enhanced synthesis of antioxidant enzymes are more thermotolerant [21].

To gain a better insight into the mechanisms involved in the development of thermotolerance in yeasts, we measured the activities of four antioxidant enzymes in the exponential-phase cells of *Y. lipolytica* before and after their exposure to heat (see table). As is evident from this table, the activity of catalase, glucose-6-phosphate dehydrogenase, glutathione reductase, and superoxide dismutase in heat-treated cells increased by 3.7, 1.6, 1.6, and 2.6 times, respectively. Similar results were obtained in experiments with *Y. lipolytica* cells exposed to oxidative stress [4]. It should be noted that stationary-phase yeast cells are more thermotolerant and contain more antioxidant enzymes than exponential-phase cells.

Thus, the yeast *Y. lipolytica* is rather sensitive to heat (45°C). The preincubation of yeast cells at moderate temperatures or in the presence of oxidants at low concentrations decreases the level of cAMP, augments the level of the antioxidant enzymes, and enhances cell tolerance to heat and oxidative stresses.

These observations can easily be understood by taking into account the fact that cAMP is a negative inducer of genes encoding antioxidant enzymes. The low level of cAMP in cells exposed to heat shock or oxidative stress is responsible for the increase in the activity of the antioxidant enzymes and for the enhancement of cell survival.

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