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EXPERIMENTAL ARTICLES

Effect of Sodium Azide on the Thermotolerance of the Yeasts *Saccharomyces cerevisiae* and *Debaryomyces vanriji*

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Abstract—The pretreatment of *Saccharomyces cerevisiae* and *Debaryomyces vanriji* with sodium azide was found to induce thermotolerance in both yeasts, whereas sodium azide used in combination with heat shock enhanced the thermotolerance of *S. cerevisiae* and substantially decreased the thermotolerance of *D. vanriji.* It is suggested that the different responses of the yeasts to sodium azide during heat shock are due to the different functional organizations of their mitochondrial apparatus.

Key words: sodium azide, thermotolerance, alternative oxidase, ATP synthetase.

The exposure of cells to elevated temperatures is known to induce their tolerance to subsequent heat shock [1]. The induction of thermotolerance is accompanied by the synthesis of heat-shock proteins (HSPs), some of which are involved in the formation of the heat-shock response of cells [2]. Chemical agents, such as ethanol, heavy metals, sodium arsenite, and amino acid analogues, can also induce the thermotolerance of cells [2]. Many of these compounds are known to cause protein denaturation; this fact led to the belief that the thermal denaturation of protein molecules is the main cause of the heat-induced death of cells [1, 2].

Mitochondria are obviously involved in the response of cells to heat shock, since the inhibitors of the mitochondrial electron transport chain and oxidative phosphorylation induce the same changes in the genetic activity of the polytene chromosomes of the salivary glands of *Drosophila* as the heat shock occurs [3].

The aim of the present work was to investigate the effect of sodium azide (NaN_3), which is known to suppress the functioning of the mitochondrial respiratory chain, on the thermotolerance of the yeasts *Saccharomyces cerevisiae* and *Debaryomyces vanriji*.

MATERIALS AND METHODS

Experiments were performed with *Saccharomyces cerevisiae* α w^+303-1B, which was kindly provided by F. Lacroute from the Center of Molecular Genetics in Gif-sur-Yvette (France), and *Debaryomyces vanriji* GK46-2, which was isolated from a hot spring (46°C) near the town of Goryachinsk (Buryatia, Russia). Yeasts were grown in YEPD medium containing (g/l) yeast extract, 5; peptone, 10; and glucose, 20. They were also grown in a minimal medium containing (g/l) glucose, 20; KH_2PO_4, 0.9; K_2HPO_4, 0.1; $MgSO_4$, 1.0; $(NH_4)_2SO_4$, 1.0; and 200 μg/l thiamine. When necessary, the media were solidified with 15 g/l agar. Cultivation was performed at 30°C (*S. cerevisiae*) and at 40°C (*D. vanriji*).

Material for inoculation was prepared by growing yeasts in liquid YEPD medium for 14–16 h at 30°C on a temperature-controlled shaker. Fresh nutrient medium was inoculated with an aliquot of the overnight culture and incubated to a culture turbidity of 0.5, which corresponded to a concentration of 2×10^7 cells/ml in the case of *S. cerevisiae* and to 1×10^8 cells/ml in the case of *D. vanriji*.

To estimate the effect of the sodium azide pretreatment of yeast cells on their thermotolerance, the cell suspensions of *S. cerevisiae* (2×10^7 cells/ml) and *D. vanriji* (1×10^8 cells/ml) were incubated in the presence of 0.15 mM NaN_3 for 30 min at 30°C. The suspensions were then diluted at least 1000-fold to lower the concentration of sodium azide, and 1-ml aliquots of the diluted suspensions containing, respectively, 10^3 and 10^4 cells/ml were transferred to test tubes and exposed to 45°C for 0, 15, 30, 45, and 60 min.

To study the combined effect of sodium azide and heat shock on cell survival, NaN_3 was added (to a final concentration of 0.15 mM) to the cell suspensions of *S. cerevisiae* (2×10^7 cells/ml) and *D. vanriji* (1×10^8 cells/ml), after which the suspensions were immediately exposed to heat shock as described above.

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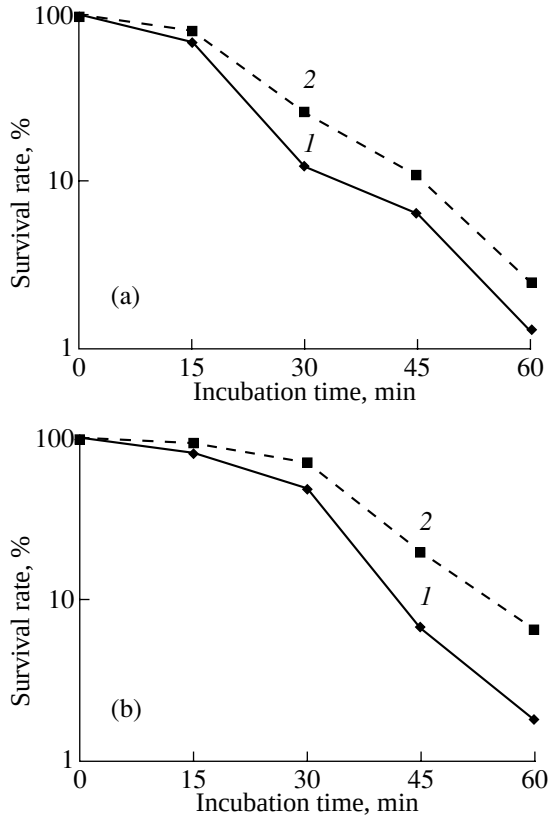

Fig. 1. Survival of (a) *S. cerevisiae* and (b) *D. vanriji* cells exposed to heat shock at 45°C either (*1*) before or (*2*) after their pretreatment with 0.15 mM sodium azide at 30°C for 30 min.

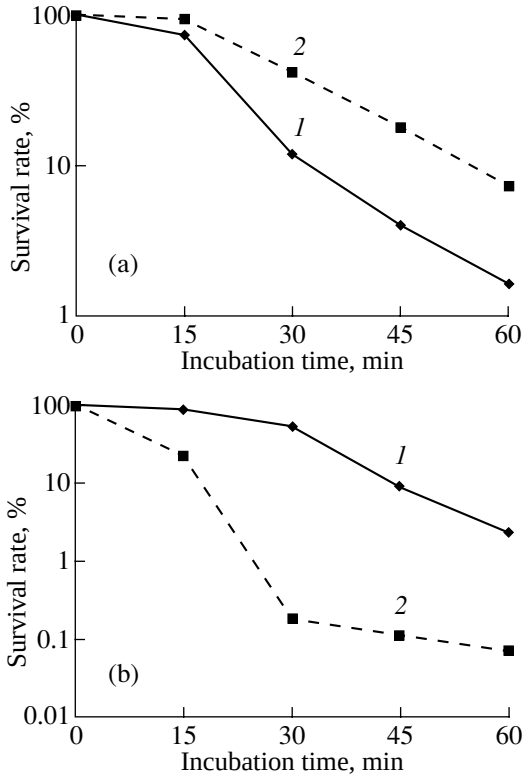

Fig. 2. Survival of (a) *S. cerevisiae* and (b) *D. vanriji* cells exposed to heat shock at 45°C either (*2*) in the presence of 0.15 mM sodium azide or (*1*) in its absence.

After heat treatment, the cell suspensions were cooled, appropriately diluted, and plated onto minimal agar medium. The plates were incubated at 30°C. The grown colonies were enumerated after 24–28 h of incubation. Cell survival was defined as the percentage of colonies grown after the respective exposure period in relation to the number of colonies grown in the control.

To estimate the respiration rate of cells, 1.4 ml of the suspension of cells grown on YEPD medium was placed into a polarographic cell kept at 30°C. Oxygen consumption was measured with a Clark-type oxygen electrode. Sodium azide and benzohydroxamic acid (BHA) were added to the polarographic cell to a final concentration of 0.15 and 2 mM, respectively. The respiration rates were expressed in nanomoles of oxygen consumed per min per 10^7 cells (*S. cerevisiae*) or per 10^8 cells (*D. vanriji*), taking into account the solubility of oxygen in water at different temperatures [4].

To assay catalase activity, yeast cells were precipitated by centrifugation at 5000 *g* for 5 min, washed thrice with K,Na phosphate buffer (pH 7.0), again precipitated, and then stored at –20°C for one day to be used for the preparation of the cell-free extract. To disrupt the cell wall, the yeast biomass was resuspended in K,Na phosphate buffer (pH 7.0), frozen in liquid nitrogen, and then ground with quartz sand. Protein fraction was separated from cell debris by centrifugation at 15000 *g* for 15 min. Catalase activity was assayed polarographically by measuring the rate of the evolution of O_2, which resulted from the decomposition of H_2O_2 by catalase [5]. Measurements were performed at room temperature using the Clark-type electrode. For this, 50 μl of the cell homogenate was placed in the polarographic cell, to which 10 μl H_2O_2 was added to a final concentration of 0.0003%. Inhibition of catalase activity was determined by adding sodium azide to the polarographic cell to a final concentration of 0.15 mM. Catalase activity was expressed in nanomoles of oxygen produced per min per mg protein. Protein was quantified by the method of Lowry *et al.* [9].

RESULTS AND DISCUSSION

The experiments performed in this work showed that the pretreatment of *S. cerevisiae* and *D. vanriji* with sodium azide made the yeasts more tolerant (sometimes by a factor of two) to the subsequent heat shock (Figs. 1a and 1b). A similar effect was induced by sodium azide in *Lactococcus lactis* [7].

However, the two yeast species under study responded differently to the combined treatment with sodium azide and heat shock: the thermostability of

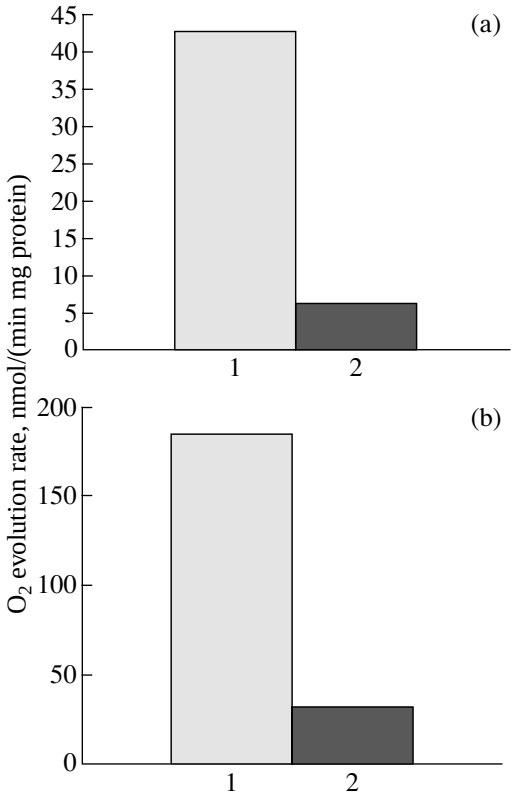

Fig. 3. The catalase activity of (a) *S. cerevisiae* and (b) *D. vanriji* cells (2) in the presence of 0.15 mM sodium azide and (1) in its absence.

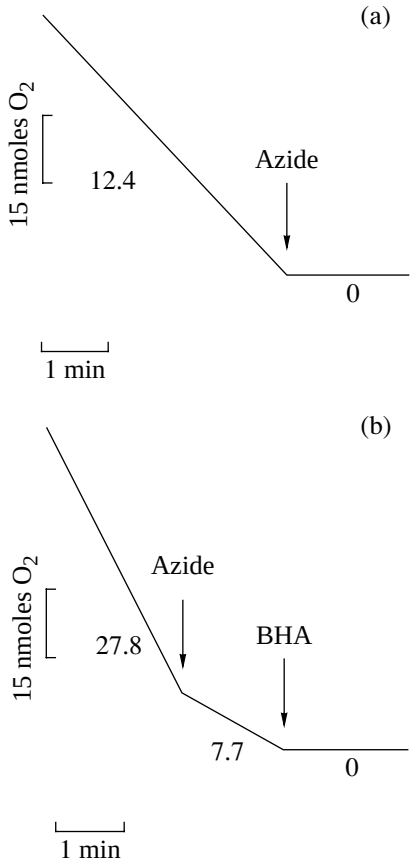

Fig. 4. Oxygen consumption by (a) *S. cerevisiae* and (b) *D. vanriji* cells. Arrows indicate the time of the addition of 0.15 mM sodium azide or 2 mM BHA. Numerals along the curves indicate the oxygen consumption rate expressed in nmoles O_2 per min per 10^7 and 10^8 cells of *S. cerevisiae* and *D. vanriji*, respectively.

S. cerevisiae increased (Fig. 2a), while that of *D. vanriji* drastically decreased (Fig. 2b). It should be noted that sodium azide produced no toxic effect on *D. vanriji* at 30°C, indicating that this inhibitor affected *S. cerevisiae* and *D. vanriji* cells in quite different ways.

It is known that heat shock induces the generation of reactive oxygen species (ROS) [8, 9], such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$), which impair cell membranes, proteins, and DNA and eventually cause cell death.

Sodium azide inhibits heme-containing enzymes, including catalase. Therefore, the 83% inhibition of the catalase activity of *D. vanriji* cells by sodium azide (Fig. 3b) should inevitably lead to an increase in the concentration of hydrogen peroxide. The latter compound may be largely responsible for the cell response to heat shock, as was suggested by Dat *et al.* [5], who studied the induction of the thermotolerance of the *Sinapis alba* mustard seedlings by another catalase inhibitor, salicylic acid.

Similarly, the decrease in the survival of the *D. vanriji* cells exposed to the combined action of sodium azide and heat shock can be explained by the inhibition of catalase activity by sodium azide and the resulting increase in the concentration of hydrogen peroxide to a toxic level. This suggestion is confirmed by the data of Lord-Fontaine and Averill, who found that the inhibition of the catalase activity of the cultured Chinese hamster ovary cells by 3-amino-1,2,4-triazole decreased their thermotolerance [9].

However, in *S. cerevisiae*, the mechanism of the action of sodium azide may be quite different, since, in spite of the fact that sodium azide inhibited the catalase activity of *S. cerevisiae* cells by 86% (Fig. 3a), the thermotolerance of this yeast not only failed to decrease, but even increased (Fig. 2a).

Inasmuch as the generation of ROS is mainly due to the mitochondrial respiration [10] and sodium azide its inhibitor, the different responses of *D. vanriji* and *S. cerevisiae* cells to this compound can be explained by the different functional organizations of mitochondria in these two yeasts.

Sodium azide completely inhibited the respiration of *S. cerevisiae* cells (Fig. 4a), which is obviously due to the inhibition of the cytochrome oxidase of this yeast. However, sodium azide only partially (by 72.3%) inhibited the respiration of *D. vanriji* cells, and only the

concurrent addition of BHA to the polarographic cell led to the complete inhibition of oxygen consumption by *D. vanriji* cells (Fig. 4b). Similar data were obtained with the use of another cytochrome oxidase inhibitor, potassium cyanide. These findings are indicative of the presence of an alternative pathway of electron transfer in *D. vanriji* cells and of its absence in *S. cerevisiae* cells [11]. The alternative pathway, which involves alternative oxidase and branches from the main respiratory chain at the level of ubiquinone, is resistant to the action of azide and cyanide but is completely inhibited by BHA [12].

It is known that, depending on whether or not alternative oxidase is present or absent in yeasts, their response to the inhibitors of electron transport chain may vary. For instance, in *Hansenula anomala*, antimycin A, which is also an inhibitor of the main mitochondrial electron transfer chain, induced more than a 100-fold increase in the concentration of superoxide anion, thereby activating electron transfer through the alternative oxidase [13]. However, the treatment of *S. cerevisiae* cells, in which the alternative oxidase is absent, with antimycin A did not change the concentration of ROS [14].

The absence of alternative oxidase in *S. cerevisiae* can probably explain the fact that, in spite of being able to induce the synthesis of HSPs, hydrogen peroxide fails to induce the thermotolerance of this yeast [15]. At the same time, the pretreatment of *Neurospora crassa*, in which alternative oxidase is present [16], with H_2O_2 led to the development of thermotolerance [17]. This suggests that the alternative oxidase may be involved in the formation of the cell response to the action of sodium azide during heat shock.

It remains, however, unclear why sodium azide substantially enhances the thermotolerance of *S. cerevisiae* cells but fails to do this in the case of *D. vanriji*. The possibility of the induction of HSP synthesis seems unlikely, since the *S. cerevisiae* cells were exposed to heat shock immediately after the addition of sodium azide.

In view of this, it is worth noting that sodium azide is also an inhibitor of the F_0F_1-ATPase of *S. cerevisiae* [18]. F_0F_1-ATPase catalyzes the synthesis of ATP at the expense of the transmembrane proton potential; however, under certain conditions, it hydrolyzes ATP and, hence, can transfer protons across membranes in the opposite direction, thereby hyperpolarizing the mitochondrial membrane and increasing the transmembrane potential mt $\Delta\psi$. Machida and Tanaka showed that the isoprenoid compound farnesol inhibits the growth of *S. cerevisiae* and increases the cellular level of ROS due to the hyperpolarization of the mitochondrial membrane [18]. Since sodium azide, but not potassium cyanide, prevented the effect of farnesol, these authors related the farnesol-induced hyperpolarization of the mitochondrial membrane to the hydrolysis of ATP by ATPase [18]. Based on these findings, we suggest that heat shock stimulates the ATP hydrolysis in *S. cerevisiae* cells, which leads to the hyperpolarization of their mitochondrial membranes. The inhibition of ATPase by sodium azide must prevent the generation of toxic ROS, thereby enhancing the thermotolerance of *S. cerevisiae* cells.

Thus, by inhibiting catalase and ATPase, sodium azide eliminates the sources of ROS in *S. cerevisiae* cells exposed to heat shock. The different effect of sodium azide on *D. vanriji* cells exposed to heat shock suggests that ROS are generated in these cells by quite a different mechanism than in *S. cerevisiae* cells or that the ATPase of *D. vanriji* is resistant to sodium azide.

The results presented in this paper are in agreement with the data obtained by Davidson *et al.* [8] and Lord-Fontaine and Averill [9], who showed that heat shock is accompanied by oxidative stress and that the generation of ROS at elevated temperatures is one of the causes of the death of cells during heat shock. The different effects of sodium azide on *D. vanriji* and *S. cerevisiae* cells exposed to heat shock may be due to the functioning of the alternative oxidase in *D. vanriji*, the different regulation of mitochondrial ATPase, and the different mechanisms of ROS generation in these two yeasts.

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REFERENCES

1. Aleksandrov, V.Ya. and Kislyuk, I.M., Cell Response to Heat Shock: Physiological Aspects, *Tsitologiya,* 1994, vol. 36, no. 1, pp. 5–59.
2. Parsell, D.A. and Lindquist, S., The Function of Heat-Shock Proteins in Stress Tolerance: Degradation and Reactivation of Damaged Proteins, *Annu. Rev. Genet.*, 1993, vol. 27, pp. 437–496.
3. Ashburner, M. and Bonner, J.J., The Induction of Gene Activity in *Drosophila* by Heat Shock, *Cell* (Cambridge, Mass.), 1979, vol. 17, no. 2, pp. 241–254.
4. *Rukovodstvo po izucheniyu biologicheskogo okisleniya polyarograficheskim metodom* (Manual on the Study of Biological Oxidation by the Polarographic Method), Moscow: Nauka, 1973.
5. Dat, J.F., Lopez-Delgado, H., Foyer, C.H., and Scott, I.M., Parallel Changes in H_2O_2 and Catalase during Thermotolerance Induced by Salicylic Acid or Heat Acclimation in Mustard Seedlings, *Plant Physiol.*, 1998, vol. 116, no. 4, pp. 1351–1357.
6. Lowry, O.H., Rosebrough, N.I., Farr, A.L., and Randell, R.J., Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem.*, 1951, vol. 193, no. 1, pp. 265– 275.
7. Boutibonnes, P., Bisson, V., Thammavongs, B., Hartke, A., Panoff, J.M., Benachour, A., and Auffray, Y., Induction

of Thermotolerance by Chemical Agents in *Lactococcus lactis* subsp. *lactis* IL1403, *Int. J. Food Microbiol.*, 1995, vol. 25, no. 1, pp. 83–94.

8. Davidson, J.F., Whyte, B., Bissinger, P.H., and Schiestl, R.H., Oxidative Stress Is Involved in Heat-Induced Cell Death in *Saccharomyces cerevisiae*, *Proc. Natl. Acad. Sci. USA*, 1996, vol. 93, no. 10, pp. 5116–5121.
9. Lord-Fontaine, S. and Averill, D.A., Enhancement of Cytotoxicity of Hydrogen Peroxide by Hyperthermia in Chinese Hamster Ovary Cells: Role of Antioxidant Defenses, *Arch. Biochem. Biophys.*, 1999, vol. 363, no. 2, pp. 283–295.
10. Longo, V.D., Gralla, E.B., and Valentine, J.S., Superoxide Dismutase Activity Is Essential for Stationary Phase Survival in *Saccharomyces cerevisiae*: Mitochondrial Production of Toxic Oxygen Species *in vivo*, *J. Biol. Chem.*, 1996, vol. 271, no. 21, pp. 12275–12280.
11. Huh, W.K. and Kang, S.O., Molecular Cloning and Functional Expression of Alternative Oxidase from *Candida albicans*, *J. Bacteriol.*, 1999, vol. 181, no. 13, pp. 4098–4102.
12. Medentsev, A.G., Arinbasarova, A.Yu., and Akimenko, V.K., Regulation and Physiological Role of Cyanide-Resistant Oxidase in Fungi and Plants, *Biokhimiya*, 1999, vol. 64, no. 11, pp. 1457–1472.
13. Minagawa, N., Koga, S., Nakano, M., Sakajo, S., and Yoshimoto, A., Possible Involvement of Superoxide Anion in the Induction of Cyanide-Resistant Respiration in *Hansenula anomala*, *FEBS Lett.*, 1992, vol. 302, no. 3, pp. 217–219.
14. Machida, K., Tanaka, T., Fujita, K., and Taniguchi, M., Farnesol-induced Generation of Reactive Oxygen Species via Indirect Inhibition of the Mitochondrial Electron Transport Chain in the Yeast *Saccharomyces cerevisiae*, *J. Bacteriol.*, 1998, vol. 180, no. 17, pp. 4460–4465.
15. Collinson, L.P. and Dawes, I.W., Inducibility of the Response of Yeast Cells to Peroxide Stress, *J. Gen. Microbiol.*, 1992, vol. 138, no. 2, pp. 329–335.
16. Li, Q., Ritzel, R.G., McLean, L.L.T., McIntosh, L., Ko, T., Bertrand, H., and Nargang, F.E., Cloning and Analysis of the Alternative Oxidase Gene of *Neurospora crassa*, *Genetics*, 1996, vol. 142, no. 1, pp. 129–140.
17. Kapoor, M., Sreenivasan, G.M., Goel, N., and Lewis, J., Development of Thermotolerance in *Neurospora crassa* by Heat Shock and Other Stresses Eliciting Peroxidase Induction, *J. Bacteriol.*, 1990, vol. 172, no. 5, pp. 2798–2801.
18. Machida, K. and Tanaka, T., Farnesol-induced Generation of Reactive Oxygen Species Dependent on the Mitochondrial Transmembrane Potential Hyperpolarization Mediated by F_0F_1-ATPase in Yeast, *FEBS Lett.*, 1999, vol. 462, no. 1–2, pp. 108–112.