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EXPERIMENTAL ARTICLES

Ultrastructural Changes in *Yarrowia lipolytica* Cells under Stress Conditions

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Abstract—Ultrastructural organization of the aerobic yeast *Yarrowia lipolytica* was studied under conditions of oxidative, heat, and ethanol stresses. It was shown that the following uniform changes in cell ultrastructure did not depend on the type of stress: enlargement of mitochondria, enhanced number and enlargement of peroxisomes, and formation of lipid granules. Similar ultrastructural changes also occurred during the transition of cells to the stationary growth phase. It was shown for the first time that accumulation of polyphosphate granules occurred as a stress response in yeasts. Moreover, numerous globular structures of unknown nature appeared on the cell wall surface under oxidative or heat stress. Under ethanol stress, the cells developed clearly marked deep invaginations of the cytoplasmic membrane. (The same changes in the cytoplasmic membrane were observed in the cells grown on ethanol.) Variations of the cell envelope structure along with the formation of polyphosphate granules were not observed in the stationary growth phase. Ultrastructural changes in the cells under stress conditions are in agreement with the previous data on survival, respiratory activity, and variations of the antioxidant systems.

Keywords: *Yarrowia lipolytica*, ultrastructure, stress.

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Comparative analysis of the cellular ultrastructural organization under various model stress conditions is a convenient methodological approach to investigation of the mechanisms of adaptation to different stresses [1, 2].

At present, there are almost no data on the study of yeast cell ultrastructure under acute stress (during a short period of time). There are only individual reports that *S. cerevisiae* after mild heat exposure (37°C, 30 min) shows partial compression of the nucleus and formation of electron-dense particles in mitochondria and cytoplasm [3].

Previous works [4–7] have shown that oxidative, heat, or ethanol stress results in variation of the physiological parameters of cells: survival, respiration, activities of the antioxidant enzyme systems, etc.

The goal of this work was investigation of the ultrastructural organization of the yeast *Yarrowia lipolytica* during adaptation to the conditions of oxidative, heat, or ethanol stresses.

MATERIALS AND METHODS

The yeast under study was *Yarrowia lipolytica* VKM Y-2378 from the All-Russian Culture Collection of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences [4]. Cultivation was carried out at 29°C in 750-ml flasks containing 100 ml of Reader medium [4] with glucose (1%) or ethanol (1%) on a shaker (200 rpm). Yeast growth was assayed by optical density (λ = 540 nm). The cells were washed with sterile distilled water and resuspended in 50 mM Tris–phosphate buffer (pH 7.0).

Stress conditions were modeled by 60-min incubation of the cells in the presence of H_2O_2 (0.5 mM) for oxidative stress, at 37°C for heat stress, and in the presence of 5% ethanol for ethanol stress. The above stress conditions were selected previously [4–7] as nonlethal in order to enhance the resistance (adaptation) of the cells.

For the analysis of ultrastructure, the cells were centrifuged at 15000 *g*; the pellet was fixed with 1.5% glutaraldehyde solution in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h, washed three times with the same buffer, and additionally fixed in 1% OsO_4 solution in 0.05 M cacodylate buffer (pH 7.2) at 20°C for 3 h. After dehydration with alcohol, the material was embedded in Epon 812. Ultrathin sections were mounted on grids, contrasted for 30 min with 3% uranyl acetate solution in 70% alcohol, and additionally contrasted with lead citrate according to Reynolds [8]. Ultrathin sections were examined with a JEM-100B

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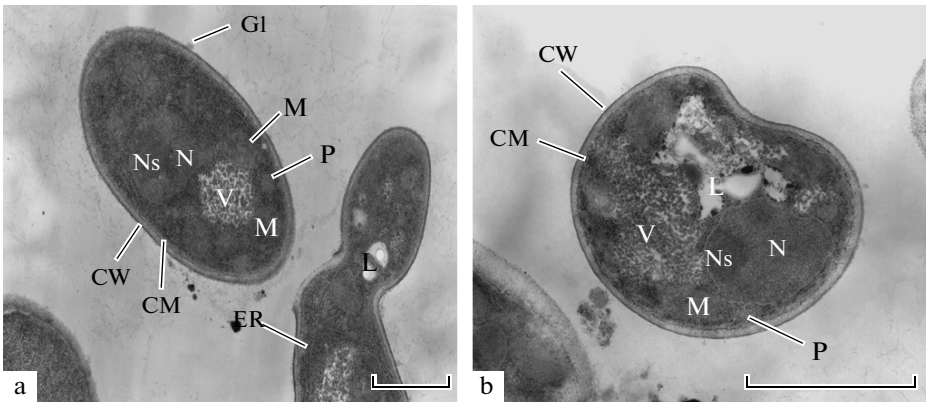

Fig. 1. Ultrathin sections of *Y. lipolytica* cells from the exponential (a) and stationary (b) growth phases. Scale bar length: 1 μm. N, nucleus; Ns, nucleolus; CW, cell wall; CM, cytoplasmic membrane; L, lysosome; ER, endoplasmic reticulum; M, mitochondrion; P, peroxisome; V, vacuole; Lp, lipids; Gl, globular structures; PP, polyphosphates; and In, cytoplasmic membrane invaginations.

electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

For X-ray microanalysis of the elemental composition, thin sections of the cells without additional contrasting were mounted on formvar-coated copper grids and vacuum-deposited with carbon at an angle of 90°. X-ray microanalysis of the sections was performed with a JEM-100CXII electron microscope (JEOL, Japan) equipped with an EM-ASID4D scan unit and a LINK-860 X-ray microanalyzer with an E5423 detector (Link-System, England) at a magnification of 20 000 and a voltage of 60 keV. The X-ray spectra were processed using the ZAF/P software package [9].

RESULTS AND DISCUSSION

As is shown in Fig. 1a (the control), *Y. lipolytica* cells in the exponential growth phase (on 1% glucose) are characterized by homogeneous structure of the cytoplasm with pronounced organelles, such as nucleus with a nucleolus, vacuole, lysosome, peroxisomes, and membranes of the endoplasmic reticulum. Mitochondria are small and located mainly in the peripheral zone of cell cytoplasm.

Previously [4–7], investigation of resistance of the yeast *Y. lipolytica* under oxidative, heat, or ethanol stresses made it possible to identify the lethal conditions and to find ways to achieve enhanced cell survival, including soft "heat hardening" and pretreatment of the cells with nonlethal doses of oxidants or ethanol. It was shown that adaptation, independently of the stress factor, resulted in a decrease of cAMP content in the cells, enhanced activities of the antioxidant enzymes (catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, and glutathione reductase) and NAD^+-dependent alcohol dehydrogenase, and emergence of an alternative pathway of electron transfer.

Besides rearrangements in the cell metabolic activity and antioxidant status under unfavorable conditions, it was natural to expect some changes in the ultrastructural organization of the cells.

Indeed, under mild stress conditions, e.g., after the action of low doses of oxidants (0.5 mM H_2O_2) (Fig. 2a) or ethanol (5%) (Fig. 2c) and "heat hardening" (37°C, 60 min) (Figs. 2, 2b), ultrathin sections revealed increased numbers of peroxisomes located along the periphery of the cytoplasm. Mitochondria under these conditions had greater sizes and a denser matrix with more cristae than did the control. After any of the above stressor impacts, large electron-transparent lipid granules were seen on ultrathin sections, which were not present in the control.

It should be noted that all these ultrastructural changes occurred also after transition of the cells to the stationary growth phase.

As was shown previously [4–7], *Y. lipolytica* cells from the stationary growth phase were more resistant to all stressors than were the cells from the exponential phase. At the same time, they possessed higher activities of the antioxidant enzymes (two- to threefold) and of the alternative oxidase (fivefold), particularly demonstrating the adaptive potential of a cell that ensured its survival.

All of the above stress conditions were characterized by a change in the cell wall surface structure. Under oxidative or heat stress, numerous globular structures of unknown nature appeared on the cell wall surface (Figs. 2a, 2c). Under ethanol stress (Figs. 2b), the cells developed distinct deep invaginations of the cytoplasmic membrane with the complete absence of globular structures on the outer cell wall surface. The same changes in the envelope organization were observed in the cells grown on ethanol (data not shown).

The above changes in the cell wall (emergence of numerous globular structures or membrane invagina-

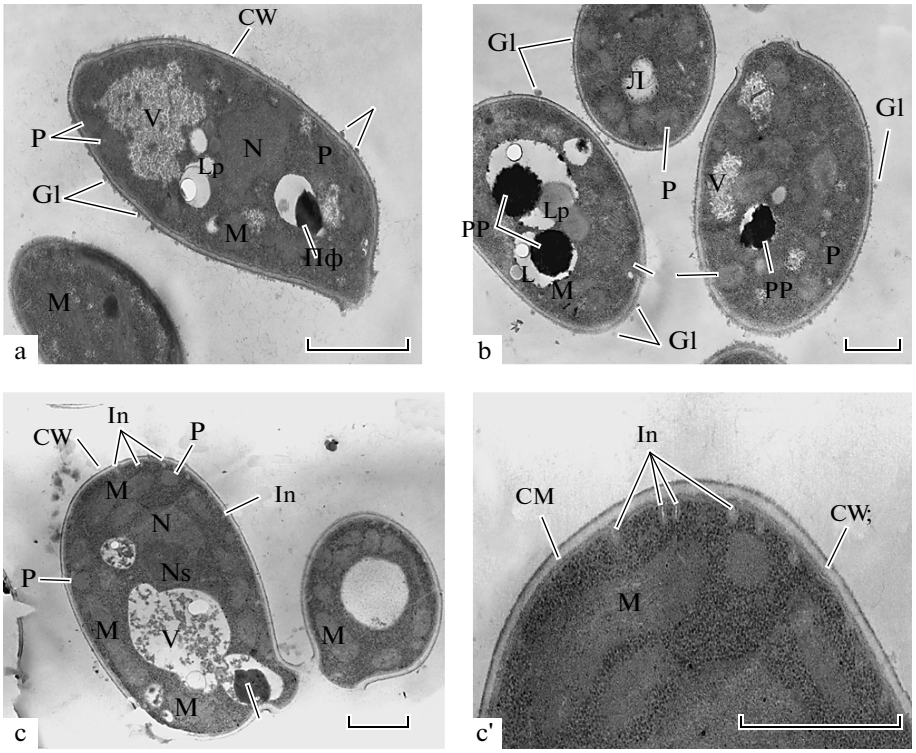

Fig. 2. Ultrathin sections of *Y. lipolytica* cells under stress conditions. 0.5 mM H_2O_2, 60 min (a); 37°C, 60 min (b); 5% ethanol, 60 min (c); and a fragment of ultrathin section c (c'). Scale bar length: 1 μm. The designations are as in Fig. 1.

tions) were not found in the cells from the stationary (both early and late) growth phase.

Moreover, it should particularly be noted that, under stress conditions, electron-dense granules emerged that exhibited high structural similarity to the intracellular polyphosphate granules described previously in bacteria from marine sediments [9].

The elemental composition of these inclusions was studied using X-ray microanalysis of thin sections. The results are presented in Figs. 3 and 4. The inclusions under consideration were shown to contain phosphorus, which is unambiguous evidence in favor of these electron-dense intracytoplasmic inclusions really being polyphosphates.

Polyphosphate granules were found both in the lysosomes and cytoplasm. The inclusions in the cytoplasm had a lower electron density and contained chlorine (Fig. 4). No chlorine was revealed in the polyphosphate inclusions localized in the lysosomes (Fig. 3). These data denote two different types of localization and the possible chemical structure of polyphosphates in yeast cells. The nature of chlorine is still unknown.

According to the literature data [9], X-ray microanalysis of the elemental composition of polyphosphates in bacteria from marine sediments showed the presence of accompanying chlorine. It was also noted that in *Desulfovibrio gigas* polyphosphate granules could contain not only polyphosphates, but also other phosphorus components: α-glucose-1,2,3,4,6-pentakis (diphosphate) [10].

Polyphosphate accumulation under stress conditions is a phenomenon well known for bacteria. It was observed in *E. coli* under nitrogen starvation and in *Helicobacter pylori* at the stage of infection [11].

The presence of polyphosphates in aerobic yeasts has not been reported previously. The peculiarities of polyphosphate metabolism and function have been fragmentarily studied in *S. cerevisiae*. One of possible function of polyphosphates is regulation of the expression of the genes encoding the diversity of enzymes responsible for cell survival under stress conditions [11]. It was also suggested [11] that inorganic polyphosphates under certain conditions could be an energy reserve of a cell.

It should be particularly noted that polyphosphate granules were not found in the cells from the stationary (early or late) growth phase. Hence, polyphosphate synthesis in *Y. lipolytica* occurred only in response to stressor impacts. This fact may be used in discussion of cell responses to stresses as a physiological element of the stationary growth phase (e.g., under substrate depletion). It should be taken into account that "suddenness" is not a factor occurring in the stationary growth phase.

Up to now, the significance of polyphosphates for aerobic yeast has not been revealed. It is probably associated with the preservation of the mechanisms of

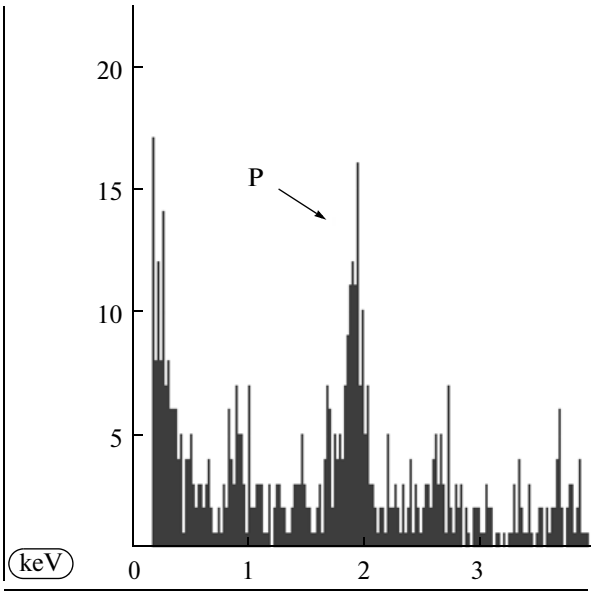

Fig. 3. X-ray spectrum of an electron-dense granule in the lysosome of *Y. lipolytica* cell (phosphorus is registered).

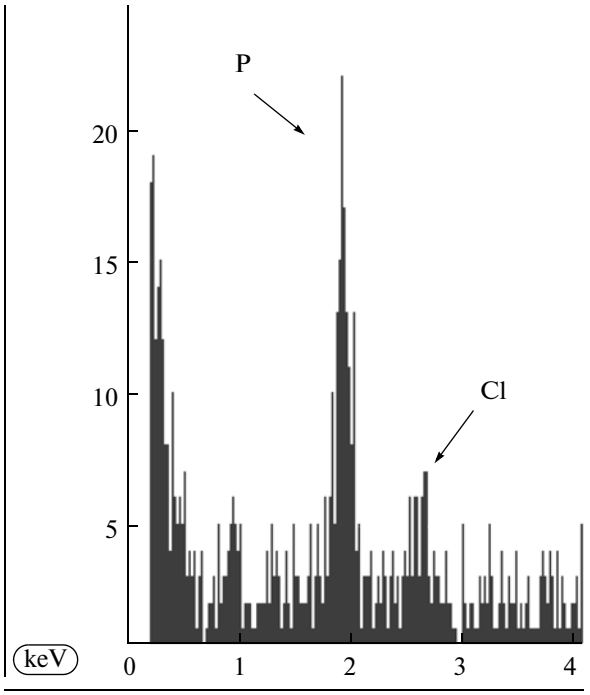

Fig. 4. X-ray spectrum of an electron-dense granule in the cytoplasm of *Y. lipolytica* cell (phosphorus and chlorine are registered).

energy provision for a cell in the course of adaptation to stress conditions.

The revealed ultrastructural changes occurring under stress conditions are in agreement with the previously obtained data on survival, respiratory activity, and variations in antioxidant systems [4–7]. The previous study of adaptation of *Y. lipolytica* to different stress factors has shown that the action of stressors leads to a decrease in the respiratory activity of cells [6]. The consequences of the disturbance of electron transfer through the respiratory chain are enhanced generation of reactive oxygen species (ROS) (at the level of ubiquinone), decreased level of cAMP, and lower ATP content. The change in the energy status of a cell is probably due to the appearance of polyphosphates and the change in the size of mitochondria. The energetic aspects of adaptation are still insufficiently studied.

The demonstrated emergence of many large peroxisomes during cell adaptation to all stressor impacts studied is in agreement with the results of the measurement of activities of the antioxidant enzymes involved in ROS detoxification [4–7]. As a defense reaction under stress conditions, the cells additionally synthesize, in particular, catalase and superoxide dismutase (SOD).

The high content of peroxisomes associated with an increase in catalase content has been shown previously for the yeasts *Yarrowia* and *Torulopsis* [12]. Moreover, catalase of the "respiratory" mutant of *Candida valida* is known to participate in ethanol oxidation [12]. The increase in the number and size of peroxisomes and the emergence of globular structures at the cell wall surface were previously noted during the growth of *Y. lipolytica* cells on hexadecane [13].

In conclusion, it should be emphasized that the revealed changes in cell ultrastructure are practically the same under all stressor impacts. Moreover, as has been shown previously [4–7], the intracellular level of cAMP in *Y. lipolytica* decreases similarly independent of the type of stress, which correlates with increased activities of the antioxidant enzymes (catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, and glutathione reductase) and NAD^+-dependent alcohol dehydrogenase, as well as with appearance of an alternative pathway of electron transfer.

The uniformity of cell response to all stress factors suggests that the defense mechanisms are unspecific or that a common center for activation of defense mechanisms exists. On the other hand, various stressor impacts may be reduced to generation of reactive oxygen species, i.e., to oxidative stress.

Thus, the process of adaptation of the yeast *Y. lipolytica* to different stressor impacts is accompanied by the following rearrangements in the ultrastructural organization of cells: enhancement of the matrix density and the mitochondrial size, increase in the quantity and size of peroxisomes, emergence of intracytoplasmic lipid and polyphosphate inclusions, and variation of the outer cell wall surface structure consisting in the development of numerous globular structures of unknown nature (oxidative and heat stresses)

or distinct deep invaginations of the cytoplasmic membrane (ethanol stress).

These results show that stress responses are manifested not only as intensification of some functions of a cell resulting in variation of the ratios of different enzymatic activities in the frame of normal growth and metabolism, but also involve additional "specialized" reactions, including the formation of new cell ultrastructures.

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