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Structural changes in the cell envelope of *Yarrowia lipolytica* yeast under stress conditions

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Draft

Abstract

The ultrastructural changes in the cell envelope of the yeast *Yarrowia lipolytica* as stress response were examined using electron microscopy technique. The formation of new cellular surface structures including membrane vesicles, pore channels and wall surface globules were shown for the first time under conditions of oxidative (endogenous and exogenous) or thermal stress. This demonstrates once again that under stress conditions the microorganisms reveal properties unknown for them before. Particularly noteworthy is the silicon accumulation, which was revealed at the surface globules with X-ray microanalysis of the elemental composition of cells' thin sections. Multi-layered plasmalemma instead of a three-layered one is also characteristic for stressed cells. The envelope modifications above were observed only as stress response and were not detected at the cells of stationary growth phase that assumes different physiological states of the yeast. A decrease in intracellular level of cAMP allows us to suppose a common factor of activating defensive mechanisms and explain the similarity of the response under different stress conditions. The data presented not only enable visualize the yeast stress response and are the supplement to diversity of adaptive reactions, they raise questions about interrelations of the stress phenomena and their functional necessity in the cell.

Key words: *Yarrowia lipolytica*, stress response, cell ultrastructure, cell envelope, biosilicification.

Introduction

In natural ecosystems, microorganisms are constantly subjected to the action of unfavourable factors of the environment. The ability to exist under extreme conditions is associated with high adaptive potential of microbial cells. In response to the action of stressors, they can activate mechanisms triggering the synthesis of enzymes, as well as defensive or signal metabolites to ensure their survival and competitive ability with respect to other species.

Adaptation mechanisms are paid attention of researchers both in terms of the role of these mechanisms in the evolutionary process and the realization of biosynthetic abilities of the cells.

In this study obligate aerobic yeast *Y. lipolytica* was chosen as a suitable model. These non conventional yeasts are of great interest, being able to utilize a variety substrates including anthropogenic pollution (e.g. oil n-alkanes, industrial wastewater *etc.*) as well as to synthesize practically useful compounds (organic acids, lipases, cytochrome c, L-lactate oxidase *etc.*) (Darvishi Harzevili 2014, Arinbasarova et al. 2014).

Earlier works, in studies of the adaptive response of the yeast *Y. lipolytica* to stress effects, showed a decrease in cells' physiological parameters, such as survival rate or respiratory activity. The disturbance of the respiratory chain in its turn was also found to result in the emergence of an alternative electron transport pathway, cyanide resistant oxidase, which enables synthesize ATP at the first point of coupling at the level of endogenous NADH dehydrogenase and maintain the oxidative activity of the cell (Biryukova *et al.*, 2008; 2009, Medentsev *et al.*, 2002). Besides, an increase in activities of antioxidant systems one way or another involved in the removal of reactive oxygen species took place (ROS) (Arinbasarova et al. 2015). There are the changes in the energetic or antioxidant status of the cells that are underlying of the tolerance and providing maintenance of survival rate.

Along with rearrangements of the energetic and antioxidant systems of the cells, under unfavourable stress conditions it would also be natural to expect the changes in ultrastructural organization of the cells.

This work aims to investigate the ultrastructural organization of the aerobic yeast *Y. lipolytica* VKM Y-2378 during its adaptation to stress conditions. Special attention is given to the structure of the cell wall, which plays an important role in the stress tolerance responsible for the maintenance of shape and integrity of the cells.

Materials and methods

Microorganism, growth and stress conditions

The yeast *Yarrowia lipolytica* VKM Y-2378 was obtained from the All-Russian Collection of Microorganisms (Russian Academy of Sciences). Cells were grown at 28°C in 750-mL shake flasks (200 rpm) containing liquid Reader's medium (100 mL) supplemented with 0.2% yeast autolysate and Burkholder trace elements. Glucose (1%) or L-lactate (2%) was used as growth substrates.

Cells from the exponential growth phase were subjected to mild stress effects: treatment with small doses of oxidants or incubation at 37°C during 20-30 min (Biryukova et al. 2009, 2011, Arinbasarova et al. 2015). Hydrogen peroxide (0.5 mM) or superoxide-generating agent 2-methylnaphthalene-1,4-dione (0.05 mM) were used as oxidants. Mild stress effects made it possible to increase the survival rate and provided stress-resistant (adapted) cells.

The conditions of endogenous oxidative stress were also modeled: yeast was grown on medium with L-lactate as the sole source of carbon and energy that was accompanied by the synthesis of L-lactate oxidase, an enzyme producing hydrogen peroxide at the first stage of L-lactate oxidation (Arinbasarova et al. 2014).

Cells in the absence of stress factors (at the exponential phase of growth on glucose), were used as a control.

Extraction and determination of cAMP

The extraction of cAMP from the cells was carried out with perchloric acid (5%); the cell suspension was mixed with perchloric acid (50%) and incubated in an ice bath. The extract was neutralized with 5N KOH with vigorous stirring and centrifuged at 15000 x g for 60 min. The supernatant was stored at -15 °C. The determination of cAMP was carried out according to a standard procedure using the cAMP assay kit (Amersham).

Electron microscopy assay

Transmission electron microscopy of ultrathin sections

Cells were centrifuged at 6000 x g for 15 min; the cell pellet was fixed in 1.5% glutaraldehyde solution in 50 mM cacodylate buffer (pH 7.2) at 4°C for 1 h, washed three times with the same buffer and fixed in 1% solution of OsO₄ in the buffer for 3 h at 20°C. After dehydration with alcohol, material was embedded in Epon 812 epoxy resin. Ultrathin sections were mounted on support grids, contrasted by 3% uranyl acetate solution in 70% alcohol and lead citrate (Reynolds 1963). The sections were examined in a JEM-100B (JEOL, Japan) transmission electron microscope (TEM) at an accelerating voltage of 80 kV.

Scanning electron microscopy

The cells separated was fixed in a 1.5% glutaraldehyde solution in the cacodylate buffer above at 4°C for 1 h, and then washed three times in the same buffer. After that, cells were fixed in 1% solution of OsO₄ in the above cacodylate buffer for 3 h at 20°C. The cells were dehydrate in a series of alcohols of increasing concentrations (from 30 up to 100%) for 20 min at each stage and then kept in *tert*-butanol for 12 h at 4°C. The specimens were freeze-dried in JFD-320 (JEOL, Japan) in accordance with the manufacturer's recommendations.

The dried specimens were mounted on aluminum disks with a diameter of 32 mm by means of current-conducting tape and sputtered with a platinum-carbon mixture in JEE-4X (JEOL, Japan) vacuum evaporator.

Scanning electron microscopy (SEM) experiments were performed with JSM-6510LV (JEOL, Japan) microscope.

Cytochemical reaction to determine the localization of enzymes degrading hydrogen peroxide

Cytochemical reaction was carried out without additional contrasting using the cells under conditions of endogenous oxidative stress (Arinbasarova et al. 2014). Cells grown on L-lactate were centrifuged at 6000 x g for 10 min and suspended at room temperature for 60 min in Tris-HCl buffer (pH 9.0), containing 50 mM 3, 3'-diaminobenzidine (2.5 mg/mL) and L-lactate (5 mM). Then cells were washed with the cacodylate buffer and kept in 1.5% glutaraldehyde solution in cacodylate buffer at 4°C for 1 h.

X-ray microanalysis

X-ray microanalysis of the elemental composition of cells' thin sections was carried out without additional contrasting using JEM-1400 (JEOL, Japan) transmission electron microscope equipped with an INCA Energy TEM 350 (Oxford instruments, UK) energy-dispersive X-ray spectroscopy (EDS).

Results and Discussion

Figure 1 presents transmission electron micrographs of ultrathin sections of stress-resistant yeast *Y. lipolytica* (after mild stress effect). As compared with the controls (Fig. 1a and b), numerous globular surface structures (GI) of unknown nature appeared on the cell wall surface after the action of low doses of oxidants (Fig. 1c) or thermal pre-treatment (37°C, 60 min) (not shown). Formation of GIs under stress conditions was also confirmed by scanning electron microscopy (Fig. 2).

X-ray microanalysis of the elemental composition of these globular structures showed the presence of silicon element (Si) (Fig. 3). These structures containing silicon were found only at

stressed cells and were absent in the controls. X-ray microanalysis revealed also the presence of oxygen in the globules implying accumulation of silicon as silicon dioxide. There was no other elements deserved any attention at the globules.

It should be noted that biosilicification of yeast above was observed at the deficiency of silicon in the environment medium. Only trace concentrations perhaps were available because of the extraction from glass flasks. Under these conditions silicon was not detected in the other compartments of the cells.

In nature biosilicification has been shown for different organisms and as a rule at high environment silicon concentrations (Jones et al. 2008, Otzen 2012). As stress response, this phenomenon has been shown only for plants (Coskun et al. 2016). Silicon accumulation by the yeasts as stress response is shown for the first time.

Uptake of different substances, for example, heavy metals, is characteristic for *Y. lipolytica* yeasts contributing to their industrial potential (Darvishi Harzevili 2014). But, the ability to accumulate silicon has not been previously known for the *Y. lipolytica* yeasts.

The study of the peculiarities of silicon accumulation and its integration in the cell is beyond the framework of this paper and is planned for the future.

Changes of the cell wall surface as a stress response can be of another sort (Canetta et al. 2009, Pillet et al. 2014). For example, an unprecedented circular structure has been observed to form at the cell surface of *S. cerevisiae* in response to a temperature stress (Pillet et al. 2014).

Variety of morphological changes in the yeast cell surface assumes various mechanisms of stress tolerance.

It was also found that under stress conditions (e.g. endogenous oxidative stress during the growth on lactate) loose electron-transparent zones in the form of pore channels with unclear limitative contours, appeared in the cell wall (Fig. 4, a-c).

Earlier, there has been nothing known in the literature about the existence of such structures as pore channels in the yeasts *Y. lipolytica*. Formation of similar (but larger and

structurally slightly different) 'canals' has been shown for some yeasts grown on oil hydrocarbons, namely, *Schwanniomyces occidentalis*, *Candidat ropicalis* and *C. maltose* (Dmitriev et al. 2016). The role of these 'canals' was assumed in connection with their possible participation in apoptosis.

We have also carried out a cytochemical reaction to determine the localization of a hydrogen peroxide-degrading activity at stressed cells and revealed multiple membrane vesicles (MVs) discretely located on the surface of the cell wall (Fig. 5). These structures were not detected on the cell surface in the absence of stress factors.

Extracellular vesicles play an important role in the biology of various organisms, including fungi, in which they are responsible for transport of molecules across the cell wall. Fungal extracellular vesicles have been shown to carry proteins, lipids, pigments, polysaccharides, and RNA and therefore may be determinant for various biological processes, including cell communication and pathogenesis (Peres da Silva et al. 2015, Joffe et al. 2016). .

The role of MV, as well as pore channels in the process of adaptation of *Y. lipolytica* is still unknown and will be examined in detail in the future.

Anyway, MVs formed under stress conditions are probably released into intercellular space. We succeeded in fixing them only because they were retained close to the cell surface by the product of oxidative polymerization of 3, 3'-diaminobenzidine. They are lost during the ordinary course of fixation.

In this regard, the question about structural reorganizations and integrity of the cytoplasmic membrane (plasmalemma) is raised. As it can be seen on ultrathin sections of the cells from exponential or stationary growth phase on glucose (control variants) (Fig. 1a, b), the plasmalemma features a three-layer structure – two electron-dense layers and one electron-transparent layer. Under stress conditions at the stage of adaptation we found it to be multi-layered (Fig. 1d). The multi-layered character of the plasmalemma perhaps indicates the phase of

active cell restructuring, as it was shown for some bacteria at the lag phase of growth (Duda et al. 2001).

A multi-layered plasmalemma instead of a three-layered one was found to take place during the process of adaption to stress conditions which indicates a phase of active restructuring of the microorganism.

In addition, the formation of multi-layer cytoplasmic membranes, as well as emergence of MVs, can presumably be related to the overproduction of membrane phospholipids during adaptation process and the occurrence of a complex type of compartmentalization in biological membranes.

It should be reminded that structural changes described above were detected using stress resistant cells (see Materials and methods). The adaption process was carried out using mild stress effects (treatment with low doses of oxidants or incubation at 37°C) to result in increase lethal doses of the stressors, thereby increasing the cell survival (Biryukova et al. 2008, 2009, 2011, Arinbasarova et al. 2015).

Thus, adaptation of *Y. lipolytica* yeast to various stress factors includes remodelling the cell envelope, namely, cell membrane modification, the formation of the new structures such as cell wall pore channels, MVs or surface globules. The emergence of the new cell structures as stress response was also shown to take place in the other compartments of *Y. Lipolytica* (Biryukova et al. 2011). E.g., the formation of polyphosphate granules was found with X-ray microanalysis in cytoplasm under conditions of oxidative, thermal or ethanol stress.

We should also emphasize that appearance of new cell structures mentioned above including polyphosphates, MVs, surface globules and channels, as well as biosilification was found only as stress response and was unknown for *Y. lipolytica* yeasts earlier.

These phenomena were not observed in the cells at the stationary growth phase (both the early and late) and took place only as a stress response. That is, the morphology of stressed yeast cells differs from that of stationary growth phase cells assuming different physiological states of

the yeast. These results are important in the comparison of cells' physiological reactions in the stationary growth phase and at the stress response, when it should be taken into account such factor as suddenness.

The identity of envelope modifications both under oxidative (exogenous and endogenous) and thermal stress should be also noted. The similarity of stress reactions of *Y. lipolytica* irrespective of the kind of stress was also shown earlier as an increase in the size of mitochondria, in the number of peroxisomes, the emergence of lipid and polyphosphate granules (Biryukova et al. 2011). Besides, the identical variations of antioxidant enzyme activities (catalase, superoxide dismutase, glucose-6-phosphate dehydrogenase, or glutathione reductase), as well as emergence of alternative electron transport way, were also found to take place under different stress conditions (Arinbasarova et al. 2015).

Mechanisms that regulate the adaptive response of cells are not well understood.

Earlier, a pronounced capability of *Y. lipolytica* to cross resistance, when one type of stress leads to develop a resistance to other stress factors was shown (Biryukova et al. 2008, Arinbasarova et al. 2015). This cross resistance along with the uniformity of stress response of yeast to various stress factors assumes a common factor of activating defensive mechanisms.

The changes in the intracellular content of cAMP, as one of the signal molecules, in response to the action of oxidants or high temperature were measured. As you can see in Fig. 6 the content of cAMP in the absence of stressors (control) remained unchanged. Under stressful conditions, after a short-term increase, concentration of this nucleotide in the cell decreased below the initial level.

Considering the features of the action of cAMP in yeasts as a negative factor of the transcription of some genes (Belazzi et al. 1991), presumably, it is the decrease in the intracellular concentration of this nucleotide that leads to the activation of defensive mechanisms.

Conclusion

Despite the apparent independence of a cell envelope, its remodeling is the part of total stress response at *Y. lipolytica*, which occurs simultaneously with the other cellular modifications, most likely in accordance with the general signal.

A decrease in intracellular level of cAMP measured allows us to assume a common center of activation of defensive mechanisms in *Y. lipolytica* yeast and explain the similarity of the stress reaction under different stress conditions (endogenous or exogenous oxidative, thermal stresses).

The ultrastructural organization of stressed cells differs from that of stationary growth phase cells. The new cell surface structure, such as membrane vesicles, wall pore channels and surface wall globules are formed at *Yarrowia lipolytica* as stress response only. This once again demonstrates that under stress conditions microorganisms reveal properties unknown for them previously. Particularly noteworthy is the accumulation of silicon, which was detected at the surface globules.

The data presented not only enable visualize the yeast stress response and are the supplement to diversity of adaptive reactions, they raise questions about interrelations of the stress phenomena and their functional necessity in the cell.

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Figure captions

Figure 1. Ultrathin sections of *Y. lipolytica* cells under stress conditions (transmission electron microscopy): a, control (exponential growth on glucose); b, stationary growth on glucose; c, d, oxidative stress; CM, cytoplasmic membrane; CW, cell wall; Gl, globular structure; M, mitochondrion; N, nucleus; P, peroxisome; PP, polyphosphates; V, vacuole.

Figure 2. Cells of *Y. lipolytica* under oxidative stress (scanning electron microscopy): a, control (exponential growth on glucose); b, oxidative stress (exponential growth on L-lactate); Gl, cell wall globular structure. Scale bar, 2 μm .

Figure 3. An X-ray spectrum of a surface globular structure of *Y. Lipolytica*.

Figure 4. Fragments (a, b, c) of ultrathin sections of *Y. lipolytica* cells under conditions of oxidative stress (transmission electron microscopy). CW, cell wall; CM, cytoplasmic membrane; C, pore channel. Scale bars, 0.1 μm .

Figure 5. The fragments (a, b) of an ultrathin sections of *Y. lipolytica* cells (oxidative stress, growth on L-lactate). Transmission electron microscopy, non-stained sections. Pr, product of cytochemical reaction (oxidative polymerization of 3, 3'-diaminobenzidine) associating with membrane vesicles (MVs) at the cell wall. Scale bars, 0.3 μm .

Figure 6. Intracellular content of cAMP in *Y. lipolytica* cells under oxidative (A) and thermal (B) stress conditions: 1 (A and B), control; 2A, 45°C (adapted cells); 3A, 37°C; 2B, 120 mM H₂O₂ (adapted cells); 3B, 0.5 mM H₂O₂.

Figure 1

109x80mm (300 x 300 DPI)

Figure 2

70x28mm (300 x 300 DPI)

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Figure 3

181x265mm (300 x 300 DPI)

Figure 4

60x20mm (300 x 300 DPI)

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Figure 5

99x119mm (300 x 300 DPI)

Figure 6

203x142mm (300 x 300 DPI)

Legend:

nucleus

mitochondrion

silicon

polyphosphates

GI - globular structure

PC - pore-canal

MCM - multilayer cytoplasmic membrane

MV - membrane vesicle