Microbiology, Vol. 69, No. 5, 2000, pp. 504–508. Translated from Mikrobiologiya, Vol. 69, No. 5, 2000, pp. 606–611.

EXPERIMENTAL ARTICLES

Different Mechanisms of the Biochemical Adaptation of Mycelial Fungi to Temperature Stress: Changes in the Cytosol Carbohydrate Composition

E. P. Feofilova, V. M. Tereshina, N. S. Khokhlova, and A. S. Memorskaya

Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117811 Russia

Received December 29, 1999; in final form, April 11, 2000

Abstract—The effect of temperature stress on the cytosol carbohydrate composition of fungi belonging to various systematic groups was investigated. In *Mucorales* representatives (subkingdom *Eomycota*, phylum *Archemycota*, class *Zygomycetes*), adaptation to hypo- and hyperthermia occurs via the regulation of trehalose synthesis, although inositol is also involved in these processes in *Blakeslea trispora*. In *Ascomycota* (subkingdom *Neomycota*), oversynthesis of mannitol and glycerol occurs under hypothermia, whereas oversynthesis of trehalose and inositol takes place under hyperthermia. *Basidiomycota* (subkingdom *Neomycota*) use two pathways of biochemical adaptation, depending on the cytosol carbohydrate composition. In the absence of sucrose, glycerol and arabitol are involved in the adaptation to hyperthermia; trehalose accumulates under hypothermic conditions (type I of regulation). Type II regulation (revealed in *Pleurotus ostreatus*) involves sucrose rather than glycerol or arabitol. The data obtained are discussed in terms of fungal systematics and phylogeny.

Key words: mycelial fungi, temperature stress, protective cytosolic carbohydrates, trehalose, mannitol, arabitol, sucrose, glycerol

The biochemical strategy of an organism's adaptation to stress is conditional on its level of organization, and this particularly manifests itself if stress is caused by temperature changes. Eukaryotes use the following strategies of protection from low temperatures:

(1) They try to avoid cold-induced stress by inhabiting niches whose temperature does not cause tissue cooling (this is typical of animals and humans).

(2) They synthesize protective substances preventing tissue fluids from freezing (this option is preferred by fish and arthropods such as spiders, insects, etc.). The mechanism of action of protectors, particularly those of a carbohydrate nature, is as follows. Hydrogen bonds form between carbohydrates and water molecules in a process which may also involve, apart from axial groups, equatorial hydroxy groups; these bonds are tighter than those among water molecules per se. Presumably, carbohydrates (sugars, sugar alcohols) can substitute water upon membrane desiccation and thereby stabilize the lipid bilayer. Based on studies with muscle microsomes, trehalose produces the strongest, and glycerol the weakest protective effect on lipid bilayers [1]. This effect is accompanied by the stabilization of cell membrane proteins and an increase in the organism's resistance to low temperatures.

(3) Plants synthesize protectors causing the vitrification of tissue fluids that form a liquid crystal structure preventing the formation of ice nuclei [2].

(4) A fundamentally different strategy used in response to temperature stress (TS) is membrane stabilization by modification of the composition of the lipid bilayer; the lipids of biological membranes may make a reversible thermotropic transition from the gel state to the liquid crystal state [3, 4]. Additional changes in the lipid bilayer, such as the cyclization of fatty-acid chains, occur in prokaryotic organisms; this is accompanied by an effective modulation of lipid bilayer fluidity [5].

Our research focused on the fungal mechanisms of adaptation to temperature-induced effects. It aimed at elucidating the TS-dependent changes in the (1) carbohydrate and (2) lipid composition of the cytosol of fungi belonging to various systematic groups. This paper confines itself to item (1). The the systematics of fungi suggested recently by Cavalier-Smith [6].

MATERIALS AND METHODS

This study used fungal strains belonging to the phyla *Archemycota* (*Cunninghamella japonica* VKM F-1204 (–), *Blakeslea trispora* VKM F-4 (–), and *Absidia coerulea* VKM F-858 (–)), *Ascomycota* (*Myceliophthora thermophila*, a strain from the Institute of Microbiology, Russian Academy of Sciences, and *Aspergillus japonicus* VKM F-2145), and *Basidiomycota* (*Pleurotus ostreatus*, a strain from the Institute of Microbiology, Russian Academy of Sciences, *Lentinus edodes*, a strain from the collection of the Department of Mycology and Algology, Moscow State University, and *Flammulina velutipes*, a strain from the Institute of Microbiology, Russian Academy of Sciences).

0026-2617/00/6905-0504$25.00 © 2000 MAIK "Nauka/Interperiodica"

Table 1. Changes in the carbohydrate composition of fungi under temperature shock (expressed in % of the total sugar content)

Species	Cultivation temperature, °C	Glycerol	Arabitol	Mannitol	Mannose	Glucose	Inositol	Sucrose	Trehalose
C. japonica	28	–	–	–	3.3	94.2	–	–	2.4
	33	–	–	–	4.2	85.2	–	–	10.6
	17	–	–	–	–	98.8	–	–	1.2
M. thermophila	42	–	–	14.4	–	36.9	14.4	–	34.3
	50	–	–	–	–	18.4	24.0	–	57.6
	26	–	–	31.4	–	47.7	2.3	–	18.6
L. edodes	26	–	30.2	17.4	–	11.6	6.9	–	33.7
	10	5.1	58.2	10.5	–	11.8	5.1	–	9.3
P. ostreatus	26	Traces	3.2	12.9	–	19.6	3.9	18.7	41.5
	33	Traces	Traces	10.2	–	15.0	3.4	30.6	40.8
	12	Traces	3.0	17.8	–	12.9	3.0	16.8	46.5
A. japonicus	26	2.3	–	19.4	–	30.3	10.1	–	37.9
	32	–	–	3.5	–	20.1	18.9	–	57.5
	14	5.8	–	28.4	–	50.2	–	–	15.6

Note: Traces implies that the amount of the sugar does not exceed 1% of the total sugar content; "–" means that the carbohydrate was not detected.

Table 2. Formation of carbohydrate protectors in the cytosol of *F. velutipes* grown at different temperatures

Variant	Cultivation temperature, °C	Total sugar content, % of dry biomass	% of the total sugar content					
			glycerol	arabitol	glucose	mannitol	inositol	trehalose
Mature primordia	10–15	19.7	Traces	52.4	Traces	Traces	Traces	47.6
Mature primordia	0–5	13.5	31.8	50.4	4.4	6.7	Traces	6.7
Mature caps (30 mm in diameter)	10–15	17.9	Traces	70.6	1.6	4.2	Traces	23.6
Mature caps (30 mm in diameter)	0–5	37.0	34.6	55.7	2.7	3.2	Traces	3.8
Stem of a mature fruiting body	10–15	29.5	Traces	67.1	2.0	5.0	0.4	25.5
Stem of a mature fruiting body	0–5	30.6	2.7	62.4	12.4	3.9	Traces	7.6

Note: Traces implies that the amount of the sugar does not exceed 1% of the total sugar content.

*The sugars in this variant include an unidentified carbohydrate (about 11%).

Fungi belonging to *Eomycota* (*C. japonica*) were grown in Goodwin medium [7]. *C. japonica* was cultivated for 24 h at 28°C, and, thereupon, some flasks were incubated at 28°C for 6 h, while the remaining ones were kept at 33 or 17°C.

Following is the medium composition (g/l) used for growing *P. ostreatus*, a representative of *Neomycota* (*Basidiomycota*) fungi: soybean flour, 7.0; $MgSO_4 \cdot 7H_2O$, 0.38; KH_2PO_4, 3.5; sunflower oil, 10; and whey, 20% of the medium volume. Cultivation was performed in 250-ml flasks containing 50 ml of medium; a shaker (200–220 rmp) was used. 4-day-old mycelium grown on wort-agar slants at 26°C was used as inoculum. The cultivation was carried out for 72 h at 26°C. Thereupon, the flasks were subdivided into three groups, incubated for 24 h at 26, 33, and 17°C. *L. edodes* and *Ascomycota* were grown as described earlier [8, 9].

M. thermophila mycelium was obtained by cultivating a spore suspension for 24 h at 42°C; the mycelium was thereupon incubated for 3 h at 50°C. A low-temperature shock was imposed by cultivating the mycelium for 6 h at 26°C. *A. japonicus* was incubated for 6 h at 32°C and for 12 h at 14°C.

Determination of the cytosol carbohydrate composition of fungal cells. Sugars were extracted with boiling water in four 20-min stages. The resulting extract was purified of proteins. It was further purified on a combined Dowex-1 (acetate form)–Dowex 50W

(H^+) column. The quantitative composition of the sugars was determined by gas–liquid chromatography, using trimethylsilyl sugar derivatives obtained from the lyophilized extract. Arabitol or α-methyl-D-mannoside (Merck) served as internal standards. The chromatography was performed on a Model 3700 gas-liquid chromatograph equipped with a flame-ionization detector and a 2 m-long-glass column with 5% SE-30 on a 70- to 90-mesh Chromaton. The temperature was programmed to rise from 130 to 270°C at a rate of 5–6 deg/min. Glucose, mannitol, arabitol, inositol, and trehalose (Merck) served as standards.

The results obtained were statistically processed using the sign criterion; the median values were calculated at $n = 3–4$ [9].

RESULTS AND DISCUSSION

From the data of Table 1,[1] it follows that the strategies of carbohydrate protector-dependent biochemical adaptation to TS vary depending on the systematic position of Fungi. Only one biochemical mechanism, based on the conversion of glucose into disaccharide trehalose, operates in the *Zygomycetes* (*Eomycota*) tested—***C. japonica*** and *A. coerulea.* In *C. japonica* and *A. coerulea*, trehalose functions as a membrane lipid stabilizer and a peculiar reserve of glucose, a highly active carbohydrate [10]. Interestingly, a further decrease in temperature (to 10°C) results in the formation of trace amounts of glycerol. Only inositol occurs in (–) *B. trispora* stylospores obtained under hypothermic conditions.

Neomycota (*Ascomycota*) fungi, such as ***A. japonica*** and ***M. thermophila,*** are characterized by a broader spectrum of cytosol carbohydrates. In addition to trehalose, they contain a number of polyols that also function as protectors [11–14]. Hyper- and hypothermic conditions tend to enhance the formation of trehalose plus inositol and mannitol plus glycerol, respectively. Curiously enough, the behavior of this system varies depending on the optimum growth temperature of the organism. Glycerol does not occur upon a low-temperature shock in ***M. thermophila,*** whose growth optimum is at 41–42°C; however, this polyol forms in the mesophile ***A. japonicus,*** which grows at lower temperatures.

The cytosol of basidiomycete fungi contains an even wider variety of carbohydrate protectors. Additional biochemical mechanisms are involved in the temperature adaptation of these fungi. Arabitol, not mannitol (in contrast to ascomycete fungi), functions as a cryoprotector in ***L. edodes.*** Like ascomycetes, ***L. edodes.*** contains glycerol under hypothermic conditions.

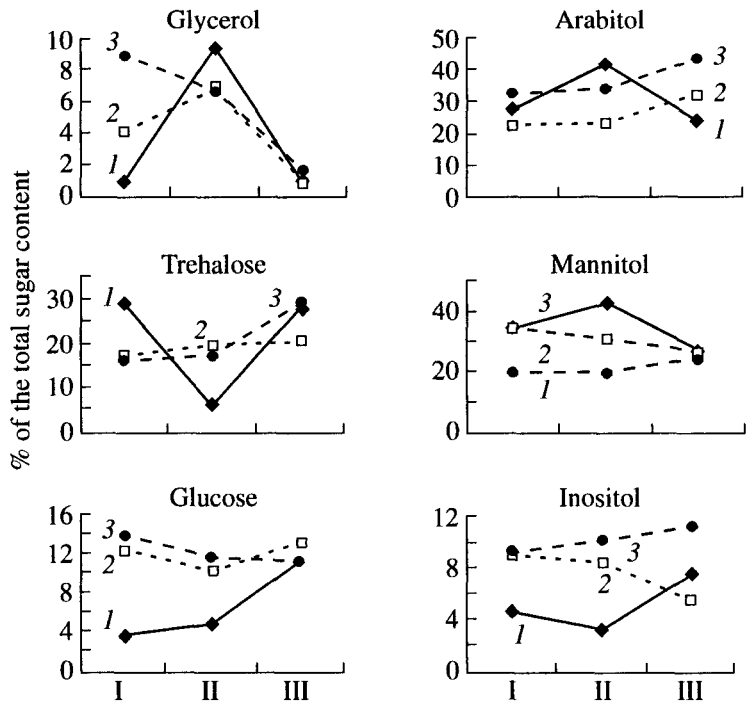
From the carbohydrate composition of the ***L. edodes*** cytosol at early stages of fruiting body formation, we inferred the sequence in which carbohydrates form (figure). For example, the glycerol amount reached its maximum, 8–9%, at the immature primordium stage in a study in which the temperature was significantly decreased (to 0°C). The level of this protector decreased subsequently, and only trace amounts were detected in a young fruiting body. Virtually no glycerol occurred at the immature primordium stage; however, it appeared at a later stage (upon primordium maturation) and subsequently disappeared at the fruiting body stage. Hence, cold shock seems to accelerate the synthesis of cytosol carbohydrates associated with particular stages of morphogenesis (e.g., the stage of fruiting body formation) and to intensify this process.

Interestingly, glycerol also occurs upon cold shock in *Ascomycetes* (but not in thermophiles [13]). It has been shown that the presence of glycerol in ***A. niger*** is correlated with the onset of particular stages of cell differentiation. For example, this polyol is virtually absent from spores [11]. These data confirm the above suggestion concerning the relationship between the presence of cytosol carbohydrates and the onset of certain differentiation stages of fungal cells. Accordingly, cold adaptation in *Neomycota* is possibly subject to regulation by the biosynthesis of carbohydrates. In *Ascomycota*, this process is chiefly controlled by glycerol, whereas arabitol is also involved in the process of adaptation in *Basidiomycota.* The polyols possibly regulate the fruiting body formation stage in basidial fungi, e.g., ***L. edodes.*** Cold shock is a prerequisite for fruiting body formation.

Of particular interest in this context is ***F. velutipes,*** which forms fruiting bodies at 0–5°C under natural conditions. The caps of this fungus contain large amounts of carbohydrates (up to 37% of the dry weight), which predominantly include two sugar alcohols, glycerol and arabitol, and the disaccharide trehalose (Table 2). The composition of protective carbohydrates varies depending on the temperature. If the fungus grows at 10–15°C in a natural habitat, then its fruiting body predominantly contains arabitol and trehalose. No glycerol occurs. Decreasing the temperature to 0–5°C results in a drastic increase in the glycerol content, a slight decrease in the arabitol content, and a significant reduction in the trehalose pool. The ratio between these carbohydrates is somewhat different in the cap and the stem. Trehalose predominantly accumulates under hyperthermic conditions (data not shown). This confirms the hypothesis (suggested by us earlier) that the synthesis of carbohydrate protectors is temperature-dependent, and that glycerol is the protector functioning at the lowest temperatures [14].

Another adaptation mechanism (Table 1) operates in the basidiomycete ***P. ostreatus*** upon a drastic decrease in the temperature: the synthesis of mannitol and trehalose is intensified. The sucrose content increases and arabitol disappears under hyperthermic

[1] In order to avoid cramming Table 1 with data, it does not contain the data obtained for all of the fungi tested; it only deals with typical representatives of systematic groups (these microbial species are in bold type in the text). The peculiarities of other tested fungi are discussed in the text.



Composition of cytosol carbohydrates during fruiting body formation in ***L. edodes*** (*1*) without a cold shock, (*2*) upon a cold shock at 10°C, and (*3*) upon a cold shock at 0°C. (I) immature primordium, (II) mature primordium, (III) young fruiting body (30 mm in diameter).

conditions. Interestingly, the pattern of temperature-dependent trehalose accumulation changed in the presence of sucrose. The trehalose amount decreased in all tested fungi, upon decreasing cultivation temperature. This may be due to the fact that trehalose can be rapidly converted into glycerol [10] at low temperatures, resulting in a decrease in the content of this disaccharide. ***P. ostreatus*** probably lacks this biochemical mechanism, and its adaptation to TS is based on the synergistic effect of trehalose and sucrose. Their ratio varies upon changing (increasing or decreasing) the temperature.

The results obtained are consistent with the following suggestion. The control mechanisms characteristic of *Ascomycota* are also involved in the adaptation to cold in basidiomycetes, whose cytosol carbohydrate pool lacks sucrose. For instance, cryoregulation is based on glycerol hypersynthesis. However, ***A. japonicus*** (*Ascomycota*) also forms mannitol, and ***L. edodes*** and ***F. velutipes*** *(Basidiomycota)* intensely synthesize arabitol.

Temperature adaptation in the sucrose-containing basidiomycete ***P. ostreatus*** is achieved using other regulatory mechanisms. Glycerol and arabitol virtually do not function as protectors under hypothermic conditions, and the mannitol and trehalose contents increase. Under hyperthermic conditions, a significant increase in the sugar content occurs, and arabitol formation is inhibited. The presence of sucrose in the pool of protector compounds actually results in the elimination of other protectors (arabitol and glycerol). Accordingly, the presence of sucrose among cytosol carbohydrates substantially changes the biochemical mechanism that regulates the adaptation to hypo- and hyperthermia in basidial fungi. The regulatory mechanism operating in this system probably resembles the mechanism characteristic of plants (e.g., *Myrothamnus flabelifolia*) [15].

The data obtained in this work (Table 1) confirm the idea that the carbohydrate composition of fungal cytosol is a reliable systematic criterion in the kingdom *Fungi* [12]. In addition, the cytosol carbohydrate composition is evidence that *Neomycota* possess a more flexible system of biochemical adaptation to temperature in comparison to other fungi. *Basidiomycota* provide a particularly interesting example. The mode of operation of their adaptation mechanisms controlling the synthesis of protector compounds varies depending on the presence/absence of the protector sucrose in the cytosol. In this respect, ***P. ostreatus*** resembles some plants, e.g. *Myrothamnus flabelifolia*, which accumulates both sucrose and trehalose during a temperature shock [15].

Importantly, the peculiar adaptation mechanisms used by some representatives of the kingdom Fungi also occur in prokaryotes, whose reaction to TS may involve the accumulation of trehalose along with prokaryote-specific protectors. Osmotic stress in bacteria results either in evacuating sodium ions from their cells and replacing them by potassium ions or in osmolyte synthesis. Most bacteria synthesize glutamate

in this situation [16]. Moderately halophilic prokaryotes and aerobic methylotrophs form glycine-betaine and ectoine, respectively [17]. Ectoine, a cyclic amino acid, also exhibits its protective properties under TS [18], in analogy to sucrose in basidiomycetes.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (grant no. 98-04-49171).

REFERENCES

1. Crowe, L.M., Mouradian, R., Crowe, J.H., Jackson, S.A., and Womersley, Ch., Effect of Carbohydrates on Membrane Stability at Low Water Activities, *Biochim. Biophys. Acta*, 1984, vol. 769, pp. 141–150.
2. Karow, A.M., Chemical Cryoprotection of Metazoan Cells, *Bioscience*, 1991, vol. 41, no. 3, pp. 155–160.
3. Nozawa, Y. and Kasai, R., Mechanism of Thermal Adaptation of Membrane Lipids in *Tetrahymena pyriformis* NT-1, *Biochim. Biophys. Acta*, 1978, vol. 529, pp. 54–66.
4. Efremenko, A.A., Lipid Metabolism and Physiological Peculiarities of Thermotolerant Yeasts, *Cand. Sci. (Biol.) Dissertation*, Moscow, 1990.
5. Russel, N.J., Mechanism of Thermal Adaptation in Bacteria: Blueprints for Survival, *Trends Biochem. Sci. Ref. Edition*, 1984, vol. 9, pp. 108–112.
6. Cavalier-Smith, T., A Revised Six-Kingdom System of Life, *Biol. Rev.*, 1998, vol. 73, pp. 203–266.
7. Feofilova, E.P., Kuznetsova, L.S., Kogtev, L.S., and Shirokova, E.A., Temperature Shock and the Lipid Composition of *C. japonica, Prikl. Biokhim. Mikrobiol.*, 1989, vol. 25, no. 3, pp. 373–384.
8. Feofilova, E.P., Tereshina, V.M., Memorskaya, A.S., Zav'yalova, L.A., and Maryshova, N.S., Changes in the Cytosol Carbohydrate Composition during the Cytodifferentiation Process in Fungi of the Order *Agaricales, Mikrobiologiya*, 1999, vol. 68, no. 3, pp. 360–365.
9. Feofilova, E.P., Gryaznova, M.V., and Sadovova, N.V., The Ability of Mannitol and Trehalose To Increase the Resistance of Mycelial Fungi to Temperature Variations, *Prikl. Biokhim. Mikrobiol.*, 1989, vol. 25, no. 5, pp. 699–706.
10. Feofilova, E.P., Trehalose, Stress, and Anabiosis, *Mikrobiologiya*, 1992, vol. 61, no. 5, pp. 741–755.
11. Witterveen, C.F.B. and Visser, J., Polyol Pools in *Aspergillus niger, FEMS Microbiol. Lett.*, 1995, vol. 134, pp. 57–62.
12. Lewis, D.H. and Smith, D.C., Sugar Alcohols (Polyols) in Fungi and Green Plants, *New Phytol.*, 1967, vol. 66, pp. 143–183.
13. Feofilova, E.P. and Tereshina, V.M., Thermophily of Mycelial Fungi as Related to Biochemical Adaptation to Temperature Stress, *Prikl. Biokhim. Mikrobiol.*, 1999, vol. 35, no. 5, pp. 546–556.
14. Feofilova, E.P., Tereshina, V.M., and Gornova, I.B., Variations in the Carbohydrate Composition of Fungal Cells as Related to the Adaptation to Temperature Stress, *Mikrobiologiya*, 1994, vol. 63, no. 5, pp. 792–796.
15. Bianchi, G., Gamba, A., Lamiroli, R., Pozzi, N., Elster, R., and Solamini, F., The Unusual Sugar Composition in the Leaves of the Resurrection Plant *Myrothamnus flabelifolia, Physiol. Plantarum*, 1993, vol. 87, pp. 223–226.
16. Galinski, E.A., Osmoadaptation in Bacteria, *Adv. Microb. Physiol.*, 1995, vol. 37, pp. 273–328.
17. Doronina, N.V., Sakharovskii, V.G., Draguk, S.V., and Trotsenko, Yu.A., Organic Osmoprotectants of Aerobic, Moderately Halophilic Methylotrophic Bacteria, *Mikrobiologiya*, 1998, vol. 67, no. 4, pp. 458–463.
18. Peters, P., Galinskii, B.A., and Trüper, N.Y., The Biosynthesis of Ectoine, *FEMS Microbiol. Lett.*, 1990, vol. 71, no. 1, pp. 154–162.