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EXPERIMENTAL ARTICLES

Adaptation of *Flammulina velutipes* to Hypothermia in Natural Environments: The Role of Lipids and Carbohydrates

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Abstract—The role of lipids and carbohydrates in the adaptation of *Flammulina velutipes* to hypothermia (5 to –5°C) in natural environments has been studied for the first time. The main changes are were found to occur in membrane lipids: the levels of sterols and glycolipids decreased, and the proportion of phospholipids with a high degree of nonsaturation (2.2) increased, which was due to predominance of two fatty acids, linoleic (35% of the total) and linolenic (50%). Phosphatidylcholine became the major phospholipid. Under hypothermic conditions, glycerol, known to have antifreeze properties, accumulated in the cell cytosol along with arabitol and trehalose. The significance of this biochemical strategy in the resistance of the fungus to temperatures below zero (i.e., to cryobiotic conditions) is discussed.

Key words: Flammulina velutipes, cryoprotectants, lipids, carbohydrates.

The basidial fungus *Flammulina velutipes* attracts the attention of researchers not only because it produces the anticarcinogenic glycoproteins EA_6 and proflavin [1] but because it retains its viability under conditions of water crystallization: this fungus is resistant to light frosts, and the formation of its fruit bodies is dependent on low temperatures [2, 3].

It is known that during cold shock, when the temperature decreases to the lower boundary of the vitality zone, fungi can use various strategies of defense: (1) regulation of the viscosity of the lipid bilayer, (2) accumulation of protective compounds of a carbohydrate nature, and (3) synthesis of heat shock proteins [2, 4–6]. Some psychrophilic fungi use only one of the above strategies [7]. A considerable difference has been shown to exist between the mechanisms of biochemical adaptation to cold stress realized in submerged cultures and those realized under conditions similar to natural ones, specifically, on soil plates. Data on the biochemical adaptation of fungi to temperatures below zero are scarce and are almost completely restricted to data on the cryoprotectant dihydrin-like polypeptides of *F. velutipes* [7].

Therefore, our aim in the present work was to unravel the mechanisms of the biochemical adaptation of *F. velutipes* to deep hypothermia by comparing the lipid and carbohydrate compositions of *F. velutipes* fruit bodies developing under natural conditions at different temperatures.

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MATERIALS AND METHODS

The *F. velutipes* fruit bodies studied experienced the following temperatures before their collection (in Bittsa forest): 7 to 15°C (optimal conditions), 3 to 5°C (variant I of hypothermia), and –5 to 1°C (light frost without snow; variant II of hypothermia). Some of the fungi were left for further observation, which showed that the fungi that had experienced temperatures below zero retained their viability, i.e., after an increase in temperature, they resumed growth. In biochemical studies, we used only fungal caps measuring 20–25 mm.

The extraction of lipids was performed according to the Folch method [8], which includes threefold extraction with a chloroform–methanol (2 : 1) mixture for 1 h at room temperature with agitation on a magnetic stirrer. For sample disintegration, we employed a freezing–thawing technique with the use of liquid nitrogen and grinding with quartz sand.

Separation of the lipids into phospholipid, glycolipid, and neutral lipid fractions was carried out on a column with silica gel L (100/160 mesh, Chemapol, Czechia) with the use of solvents differing in their degree of polarity [9]. The composition of the neutral lipids (NLs) and phospholipids (PLs) was analyzed by ascending thin-layer chromatography on glass plates covered with KSKG silica gel (Lyarne Kalur, Estonia). For the separation of the NLs, a hexane–ethyl ether–acetic acid (85 : 15 : 1) solvent system was used. For the separation of the PLs, two solvent systems were successively applied in one direction: (1) hexane–ethyl ether–acetic acid (85 : 15 : 1) and (2) chloroform–methanol–

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Variations in the composition of the neutral lipids and phospholipids found in *F. velutipes* basidiomes

Temperature, °C	Lipids, % of dry biomass	NLs : GLs*** : PLs, % of total lipids	NLs*, % of total							PLs**, % of total		
			MAGs	DAGs	Ss	FFAs	TAGs	SEs	Cs	PC	PS	PEA
(7–15)	6.8	51.0 : 20.5 : 25.5	1.7	15.3	37.7	1.4	36.5	Traces	7.4	49.8	10.7	39.5
(3–5)	5.5	44.4 : 23.8 : 31.8	1.3	20.4	32.2	9.2	30.1	3.5	3.3	68.5	2.8	28.7
–(1–5)	3.7	51.8 : 19.9 : 28.3	5.2	4.2	28.0	4.4	36.8	6.6	14.8	64.5	9.7	25.8

* Neutral lipids: MAGs, monoacylglycerides; DAGs, diacylglycerides; Ss, sterols; FFAs, free fatty acid; TAGs, triacylglycerides; SEs, sterol esters; Cs, carbohydrates.
** Phospholipids: PC, phosphatidylcholine; PS, phosphatidylserine; PEA, phosphatidylethanolamine.
*** Glycolipids.

acetic acid–water (25 : 15 : 4 : 2). A 50- to 100-μg lipid sample was applied to the plate. The chromatograms were treated with 5% sulfuric acid in ethanol and then heated at 180°C until the appearance of spots. To identify the PLs, we used individual standards and an extract of pig brain phospholipids, as well as qualitative reactions with ninhydrin (to reveal the presence of amino groups) and Dragendorff reagent (to reveal choline-containing PLs). The NLs were identified using individual standards: mono-, di-, and triacylglycerides, free fatty acids, ergosterol, and carbohydrates (Sigma). In order to obtain quantitative data, the chromatograms were scanned with a Shimadzu dual-wavelength flying-spot scanner CS-9000.

Determination of NL, PL, and glycolipid fatty acids was performed on a Model 3700 gas–liquid chromatograph equipped with a flame-ionization detector and a 2-m glass column filled with 17% DEGS on a Chromosorb W-AW ΔMGS-HP (80–100 mesh) solid carrier. The chromatography was performed isothermically at a column temperature of 170°C. The standards used for identification were individual methyl esters of fatty acids (Sigma). Some analyses were performed on a Hewlet Packard chromatograph equipped with a 50-m FFAP capillary column that had an inner diameter of 0.2 mm. Nitrogen, at a flow rate of 0.5 ml/min, was used as the carrier gas. The temperature was programmed to rise from 150 to 200°C at a rate of 5°C/min and then from 200 to 230°C at a rate of 2.5°C/min.

In order to determine the carbohydrate composition of the cytosol in the fungal spores, sugars were extracted with boiling water four times for 20 min. The removal of proteins [10] and charged compounds from the extract obtained was performed using a combined column with Dowex-1 (acetate form) and Dowex 50W (H^+) ion-exchange resins. After lyophilization, trimethylsilyl sugar derivatives were obtained [11], which were analyzed by gas–liquid chromatography using α-methyl-D-mannoside (Merck) as an internal standard. The chromatography was performed on a Model 3700 equipped with a gas–liquid chromatograph equipped with a flame-ionization detector and a 2-m glass column with 5% SE-30 on Chromaton (70–90 mesh). The temperature was programmed to rise from 130 to 270°C at a rate of 5–6°C/min. Glucose, mannitol, arabitol, inositol, and trehalose (Merck) were used as standards.

RESULTS AND DISCUSSION

The psychrophilic fungus *F. velutipes* is able to develop in a wide temperature range: vegetative mycelium has a growth optimum at 20–22°C, whereas the formation of fruit bodies requires cold shock or a decreased temperature (10–16°C), at which, even in complete darkness, the production of fruit bodies with rudimentary caps (pinhead fruit bodies) occurs [2]. It was established that, without a temperature decrease, light does not induce fruiting, but it is needed for further development of a fungal cap. These data suggest that a decreased temperature produces a morphogenetic effect; i.e., it is necessary for the realization of the *F. velutipes* life cycle. Therefore, this fungus is an interesting model for studies of biochemical adaptation to hypothermia.

At an optimal temperature, the total content of lipids in *F. velutipes* caps reaches 6.8% of their dry mass; at temperatures below zero, it decreases almost twofold. Polar lipids make up 49–55% of the total lipids, and phospholipids are present in larger amounts (26–32%) than glycolipids (20–24%) (table). The main change that occurs in the composition of neutral lipids under hypothermia is an increase in the proportion of hydrocarbons and sterol esters against the background of a decrease in sterol content. The phospholipid composition exhibits a considerable change in the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PEA), specifically, from 1.25 under optimal conditions to 2.5 under hypothermia.

A peculiarity of the fatty acid composition of the fungus is the predominance of $C_{16:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ in the fraction of neutral lipids and glycolipids and the prevalence of $C_{18:2}$ and $C_{18:3}$ in the phospholipids. Under hypothermia, an increase in the proportion of $C_{18:3}$ in the neutral lipids occurs against the background of a decrease in the $C_{18:1}$ level and a small amount of $C_{21:1}$ appears (Fig. 1); however, the nonsaturation degree changes insignificantly (1.33, 1.41, and 1.40, respectively). The fatty acids of the phospholipids exhibit the highest nonsaturation degree, since the most prominent among them are $C_{18:2}$ and $C_{18:3}$, which make up 75% of the total fatty acids at temperatures above zero and up to 85% under hypothermia. It is noteworthy that, even at optimal temperatures, the non-

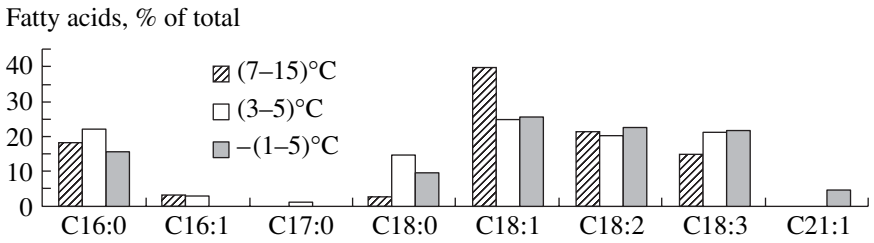

Fig. 1. Fatty acid composition of neutral lipids of the caps of *F. velutipes* basidiomes under hypothermia.

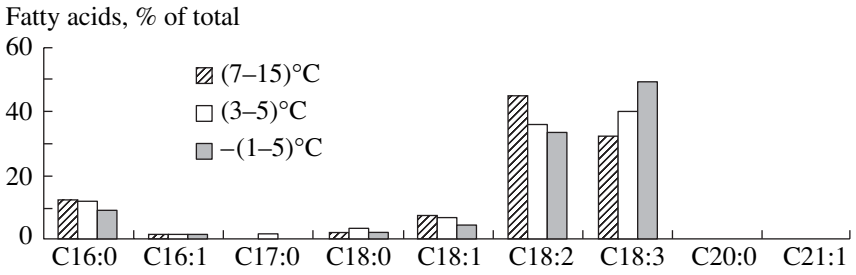

Fig. 2. Fatty acid composition of the phospholipids in the caps of *F. velutipes* basidiomes under conditions of hypothermia.

saturation degree of the phospholipids is rather high (1.95). At temperatures below zero, it reaches a value (2.20) that makes *F. velutipes* a record-holder among fungi. The main changes occurring in the phospholipid fatty acids are an increase in $C_{18:3}$ (to 50%) against the background of a decrease in $C_{18:2}$ (Fig. 2). In contrast to the phospholipids, the glycolipids increase their nonsaturation degree under variant I hypothermia (1.01 and 1.54) but decrease it at temperatures below zero (to 0.98) due to a twofold decrease in linolenic acid, a 1.5-fold decrease in linoleic acid, and the disappearance of $C_{20:1}$ and $C_{21:1}$ (Fig. 3). Interestingly, deep hypothermia results in an increase in the proportion of fatty acids with a lesser chain length, among which palmitic acid is the most prominent.

Thus, we found that, under the conditions of hypothermia, it is the membrane lipids, whose main components are phospholipids, glycolipids, and sterols, that undergo considerable changes. An increase in the proportion of phospholipids occurs against the background of a decrease in the proportions of sterols and glycolipids. In the composition of the phospholipids, the proportions of phosphatidylcholine and linolenic acid increase, resulting in an increase in the nonsaturation degree. Thus, it is the phospholipids that should be considered as the main modulators of lipid bilayer viscosity. An additional effect may be produced by the increase in the proportion of short-chain fatty acid in the glycolipids and the decrease in the sterol content. It is of interest to consider these data from the viewpoints of two hypotheses put forward to explain the adaptation of membranes to temperature variations. The first hypothesis assumes that membrane viscosity is retained due to changes in the nonsaturation degree

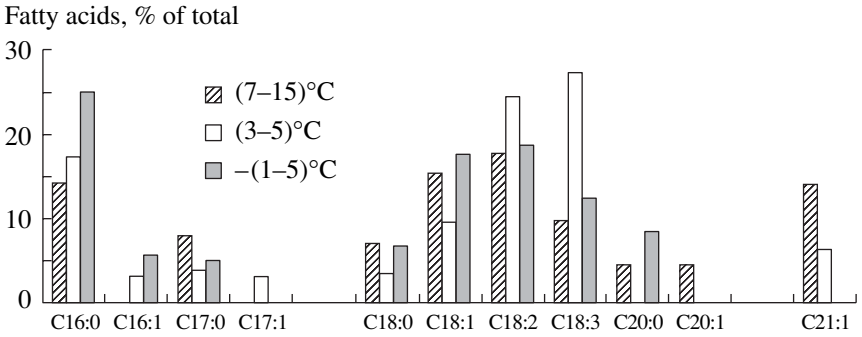

Fig. 3. Fatty acid composition of the glycolipids in the caps of *F. velutipes* basidiomes under conditions of hypothermia.

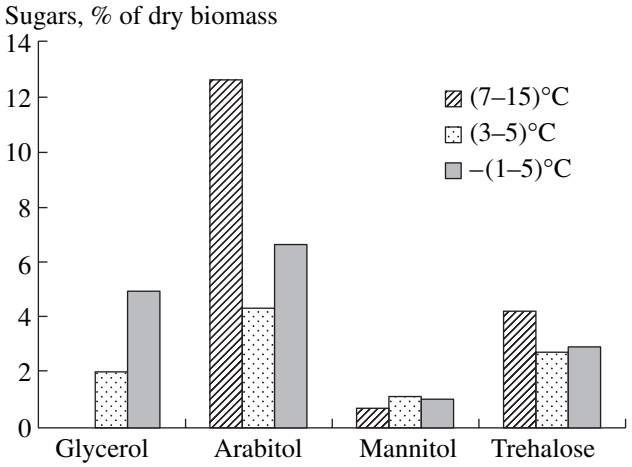

Fig. 4. Carbohydrate composition of the cytosol in the caps of *F. velutipes* basidiomes as dependent on temperature.

of the fatty acids [12]. The second hypothesis attaches particular significance to change in the balance of membrane-stabilizing and membrane-destabilizing lipids [13]. Our results are in good agreement with Hazel's hypothesis: we observed an increase in the membrane-stabilizing phosphatidylcholine and an increase in the content of polyunsaturated fatty acids ($C_{18:2}$ and $C_{18:3}$). Polyunsaturated fatty acids are known to contribute, due to their spatial organization, to the stabilization of the lipid bilayer, whereas their effect on the viscosity of the lipid component of the membranes is weaker than that of $C_{18:1}$ [13]. Noteworthy is the high content of linolenic acid that we recorded in the phospholipids (up to 50% of the total). This is unusual for basidial fungi, whose characteristic fatty acid is linoleic acid (a fact used in chemosystematics) [5]. For example, upon cold shock (5°C), the total lipids of *Lentinula edodes* contain up to 86% linoleic acid, whereas linolenic acid is absent [6]. Under optimal temperature conditions, soluble carbohydrates in the cytosol of *F. velutipes* caps made up 15.3% and were represented by trehalose and the two polyols arabitol and mannitol. Arabitol was found to be the predominant sugar (81% of the total sugars), which is in agreement with data obtained under laboratory conditions at 15–17°C [14, 15]. Characteristic features of the carbohydrate composition under variant I hypothermia were the appearance of a new polyol, glycerol, which made up 20% of the total sugars, a more than threefold decrease in the arabitol content, and a 1.5-fold decrease in the trehalose content. Temperatures below zero caused a 1.5-fold increase (as compared to variant I hypothermia) in the total content of carbohydrates. The content of glycerol and arabitol increased 2.5- and 1.5-fold, respectively, whereas the content of trehalose did not change (Fig. 4).

Thus, our data on the carbohydrate composition show that, under hypothermia, *F. velutipes* accumulates glycerol, which is known to be the most efficient compatible solute [7, 16]. Glycerol can decrease the temperature of water crystallization in the cells and exhibits the weakest inhibitory effect on enzymatic activities. The accumulation of glycerol under hypothermia distinguishes fungi from plants, which accumulate disaccharides and monosaccharides, while being a feature that is shared with some animals (in insects, glycerol is the main cryoprotectant, whereas special peptides are major cryoprotectants in fish [17]). In addition, under hypothermia, a decrease in the lipid content occurs in *F. velutipes* and the level of carbohydrates rises, which allows one more analogy with insects to be drawn. It should be emphasized that the synthesis of glycerol in *F. velutipes* is not accompanied by a decrease in the content of trehalose, indicating that this sugar also plays an important role in membrane protection under the conditions of water crystallization. The accumulation of glycerol and arabitol indicates that the enzymes of the glycolytic and pentose-phosphate pathways are active under conditions of deep hypothermia, which agrees with the published data [18]. Recently, it was established that, under cold shock, 22 proteins are synthesized in *F. velutipes*; however, only four of them are necessary for fruiting to occur [2]. The changes found to occur in the composition of *F. velutipes* cells allow an apparently paradoxical supposition to be made: the induced proteins have temperature optima corresponding to cold shock. However, taking into account that cold shock induces fruiting and, in another xylotrophic fungus, *Lentinula edodes*, activates carbon and nitrogen metabolism [6], we arrive at the conclusion that the regulation of metabolism by decreased temperature is an evolutionary acquisition of fungi that allowed them to occupy their ecological niche.

It should be noted that differences exist between the carbohydrate compositions of the cytosols of psychrophilic, mesophilic, and thermophilic fungi. Earlier, we showed that inositol is the major polyol of the thermophilic fungus *Myceliophthora thermophila*, whereas mannitol and arabitol are the major polyols in mesophilic and psychrophilic fungi, respectively [4]. Under thermal stress, the carbohydrate composition of mesophilic fungi is similar to that of thermophiles, whereas, under cold shock, arabitol appears, which is characteristic of psychrophiles growing under optimal conditions. In the present work, we show that, after a temperature decrease to 3–5°C, glycerol appears. Thus, a decrease in temperature results in the appearance of polyols with a lower molecular weight. These facts allow us to suggest that the composition of protective compounds is temperature-dependent, i.e., that polyols show a certain specificity with respect to temperature. Interestingly, trehalose, which is a universal fungal protective disaccharide, exhibits another accumulation pattern: under thermal stress, it is accumulated in all fungi, irrespective of whether they are psychrophilic, mesophilic, or thermophilic. In addition, our study showed that the content of trehalose does not decrease under deep hypothermia; apparently, trehalose is a necessary thermoprotectant at the boundaries of the vitality zone.

The present study yielded data that are of interest from the viewpoint of choice of the strategy of biochemical adaptation. Thus, in the psychrophilic fungus *Hummicola marvinii*, cold shock barely changes the nonsaturation degree of the fatty acids contained in it. This fungus uses only glycerol accumulation as an adaptation mechanism. In another fungus, *Geomyces pannorum*, considerable changes in the fatty acid composition of its polar lipids and in the nonsaturation degree occur, whereas the carbohydrate composition shows little variation [7]. *F. velutipes*, studied in the present work, simultaneously employs several strategies of biochemical adaptation: an increase in the content of cryoprotective polyol glycerol to protect the cytosol macromolecules; a rise in the level of phosphatidylcholine to stabilize the membranes; and an increase in the proportion of linolenic acid in its phospholipids to decrease the temperature of phasic transition and stabilize the liquid-crystalline phase of its lipid bilayer. In addition, this fungus is known to synthesize cryoprotective dihydrin-like polypeptides, which should be considered one more strategy of defense [19].

The present study did not reveal any significant differences in the biochemical adaptation mechanisms operating under hypothermia of different depths. It should be noted that the onset of below-zero temperatures in this study followed a period of low, but above-zero temperatures, which could favor adaptation and promote survival at temperatures below zero. From a theoretical viewpoint, a phenomenon deserving attention is the state of compelled dormancy (termed *quiescence*) caused by the pressure of various stress factors [17]. When the role of such a factor is played by temperature, decreasing to the sublethal values of 3–10°C, which means that an organism has to cope with water crystallization, the life under such conditions is termed *cryobiosis*. In poikilothermic organisms, cold numbness sets in after profound physiological preparation and is accompanied by a decrease in the intensity of metabolism and accumulation of cryoprotective compounds. The present study showed that *F. velutipes* has a potential capacity for living under the conditions of cryobiosis, since its cryoprotective compounds are analogous to those of animals. In our further laboratory studies, we plan to investigate this phenomenon in detail.

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