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EXPERIMENTAL ARTICLES

Dormant Cells in the Developmental Cycle of *Blakeslea trispora*: Distinct Patterns of the Lipid and Carbohydrate Composition

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Abstract—We revealed differences in lipid and carbohydrate composition between cells of mucorous fungi during endogenous and exogenous dormancy. Endogenous dormancy (zygospores) is characterized by high contents of phosphatidylcholine (about 70% of the total phospholipids) and triacylglycerol (over 90% of the total neutral lipids). By contrast, exogenous dormancy (sporangiospores) is accompanied by elevated amounts of sterols, sterol esters, and free fatty acids, which account for over 70% of the total neutral lipids. We established for the first time significant differences in the phospholipid composition between sporangiospores obtained from stylosporangia and sporangioles. Based on the data obtained, we regard the retardation of life-sustaining activities as a biochemical adaptation based on the dormancy state. We also discuss the taxonomical position of *Blakeslea trispora.*

Key words: endogenous and exogenous dormancy, biochemical adaptation, spores, carbohydrates, lipids, *Blakeslea trispora.*

In contrast to other representatives of *Archemycota*, mucorous fungi display a peculiar pattern of sexual reproduction characterized by the involvement of heterothallic (+) and (–) strains and a complex hormonal regulatory system controlling zygote formation [1, 2]. The spores of *Mucorales* resulting from asexual and sexual reproduction differ in their dormancy duration, dormancy pattern, and survival and germination rates during storage. *Blakeslea trispora* sporangiospores (exogenous dormancy) germinate whenever water is available, while zygospores (endogenous dormancy) can exist for a long time without germination; their germination occurs in response to an internal signal and is subject to control of a cytoplasmic regulatory system. Prerequisite to this process is a complex combination of external factors. Owing to the multifactor dependence of the regulatory mechanisms involved in the termination of zygote dormancy, reproductive cells only seldom have a chance to germinate [3], in contrast to sporangiospores, whose germination depends on exogenous factors only (primarily temperature and humidity).

The transition of fungal cells to the state of dormancy is an adaptive process that enables them to survive under unfavorable conditions. Biochemical adaptation of cells to stress primarily involves lipid composition changes [4], although the composition of protective cytosol carbohydrates that prevent lipid bilayer degradation [5] is of considerable importance as well. Therefore, research on the lipid and carbohydrate composition of dormant *Mucorales* cells (specifically, *Blakeslea trispora*) is expected to (i) yield new information on the differences between exogenous and endogenous dormancy, (ii) clarify the question why cells in the state of exogenous dormancy can germinate comparatively rapidly, and (iii) help us find out whether the relatively long period of endogenous dormancy is related to peculiarities of the biochemical composition of the dormant cells. Our studies concentrated on these above points.

MATERIALS AND METHODS

This work used *Blakeslea trispora* VKM F-989 (+) and VKM F-987 (–).

We harvested two types of *B. trispora* spores. Sporangiospores obtained from stylosporangia (sporangia with columns containing numerous spores) and from sporangioles (sporangia lacking columns and containing few spores) will be referred to hereinafter as ST and SS, respectively. The formation of either ST or SS can be considered in terms of temperature adaptation (stylosporangia form at higher temperatures). Therefore, we used a potato–carrot medium to obtain SS, and the culture was incubated at 25–26°C for 10 days. The same medium was used to obtain ST, but the culture was grown at 29–30°C [10]. To harvest sporan-

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giospores, the culture was treated with water. They were separated from the mycelium with a nylon sieve, centrifuged at 10000 *g*, washed with water, and lyophilized. All these steps were performed at 4°C.

To obtain *B. trispora* zygospores, mycelium fragments of the (+) and (–) strains were placed on the opposite sides of a petri dish with malt agar. Cultivation was carried out for 14 days at 26–28°C in the dark. The resulting dark zone was separated with a scalpel and removed with tweezers. The zone was carefully disintegrated in a distilled water in a mortar with a pestle, producing a suspension containing zygospores, fragments of the substrate mycelium, and suspensors. The suspension was passed through a nylon filter to obtain a zygospore-containing filtrate with small fragments of hypha and suspensors, which were removed by the following method. We added large quantities of water and incubated the mixture for 10 min to precipitate the zygospores to the bottom of the vessel. Thereupon, the water was removed by decantation, and the procedure was repeated several times until the water became transparent. Using this method, we obtained a zygospore fraction with a purity of 95–97%. The zygospores were lyophilized and stored at –10°C.

Lipid extraction was performed three times (each session lasted 1 h) by the Folch method [6], using a chloroform–methanol (2 : 1) mixture that was stirred on a magnetic mixer. Spores were disrupted by freezing in liquid nitrogen and subsequently thawing them and disintegrating them with quartz sand. The preparation was examined under a light microscope to ensure complete destruction.

The total lipid fraction was separated into the phospho-, glyco-, and neutral lipid fractions on a silica gel L column (100/160 mesh, Chemapol, Czech Republic), using solvents with different degrees of polarity [7]. The composition of neutral lipids and phospholipids was analyzed using upward thin-layer chromatography on glass plates with KSKG silica gel (Lyaene Kalur, Estonia). Neutral-lipid separation was achieved with a hexane–diethyl ether–acetic acid (85 : 15 : 1) solvent system. Phospholipids were separated using two consecutive unidirectional solvent systems: (1) hexane–diethyl ether–acetic acid (85 : 15 : 1) and (2) chloroform–methanol–acetic acid–water (25 : 15 : 4 : 2). 50–100 μg of lipids were applied to a plate. The chromatograms were sprayed with 5% sulfuric acid in ethanol and heated to 180°C to develop the blots. To identify phospholipids, we employed individual markers, pig brain phospholipid extract, and qualitative tests with ninhydrin (for amino groups) and the Dragendorff reagent (for choline-containing phospholipids). Neutral lipids were identified using individual markers: mono-, di-, and triacylglycerols; free fatty acids; sterols (ergosterol); and hydrocarbons (Sigma). Quantitative data were obtained by scanning the chromatograms with a Shimadzu dual-wavelength flying-spot scanner (CS-9000).

B. trispora zygo- and sporangiospores were consecutively destroyed by freezing and thawing, using liquid nitrogen, and then by grinding with quartz sand. The carotenoids were extracted with acetone 3–4 times to achieve complete decolorization of the suspension. The extract was separated by passing the suspension through a glass filter. The carotenoids were subsequently transferred to the hexane phase using a separation funnel and passed through a water-free Na_2SO_4 layer. The absorption spectrum was recorded in the 350–530 nm range with an SF-56 spectrophotometer (LOMO, Russia). The total carotenoid content was calculated according to [8]. Thin-layer chromatography on aluminum dioxide was used to determine the carotenoid composition. The carotenoid extract was concentrated by evaporation and applied, as a 20-mm-wide band, on an Al_2O_3 plate (with the third humidity level according to Brockman). Chromatography was performed in an acetone–hexane (1 : 49) system that contained the antioxidant butylhydroxytoluene to prevent carotenoid oxidation. The carotenoids were eluted from Al_2O_3 with the hexane containing 1% ethanol. The absorption spectra of the resulting eluates were recorded. The carotenoids were identified using crystalline β-carotene and lycopene (obtained from the Uralbiofarm Joint-Stock Company) as markers and by recording the absorption maxima in three solvents (hexane, acetone, and chloroform) and comparing our data with those available in the literature [9]. The extinction values used to determine the amounts of lycopene, β-carotene, and γ-carotene were 3450, 2505, and 3100, respectively [9].

To determine the cytosol carbohydrate composition of zygo- and sporangiospores, we extracted sugars with boiling water for 20 min; this procedure was repeated four times. The spores were isolated by centrifugation. Proteins were removed from the resulting extract [4]. The carbohydrate extract was additionally purified using a combined column with the DOWEX-1 (the acetate form) and DOWEX 50W (H^+) ion-exchange resins.The quantitative composition of sugars was determined by HPLC, using the trimethylsilyl sugar derivatives obtained from lyophilized extract [10]. Arabitol or α-methyl-D-mannoside (Merck) served as internal standards. We employed a Model 3700 gas-liquid chromatograph equipped with a flame-ionization detector and a 2-m glass column with 5% SE-30 on 70–90 mesh Chromaton. The temperature was programmed to increase from 130 to 270°C at a rate of 5–6 deg/min. Glucose, mannitol, arabitol, inositol, and trehalose (Merck) were used as standards.

The results were statistically processed using the median method [11].

RESULTS

The data of Table 1 point to significant differences between *B. trispora* cells during exogenous (sporangiospores) and endogenous (zygospores) dormancy

Table 1. Lipid composition of sporangio- and zygospores of *B. trispora*

Spore type	Lipids, % of dry biomass	Lipids, % of total lipids		
		neutral lipids	phospho-lipids	glycolipids
SS (–)	6.1	37.0	40.9	22.1
ST (–)	7.4	38.3	47.1	14.6
Zygospores	40.1	91.1	6.3	2.6

with respect to the lipid content and composition. Sporangiospores contained considerably less lipids than zygospores; this difference was at least sixfold.

B trispora sporangiospores also differed in their lipid composition from zygospores. Zygospores were dominated by neutral lipids, whereas sporangiospores predominantly contained glyco- and phospholipids, although their neutral-lipid content was also sufficiently high (Table 1).

We established the following peculiarities in the phospholipid composition of cells with different dormancy types (Fig. 1): (1) phosphatidylcholine (PC) is the predominant species in the zygospore phospholipids; zygospores are also characterized by the presence of lysophosphatidylcholine (LPC), while the phosphatidylethanolamine (PEA) content is below 10%; (2) PC also dominates the phospholipids of SS, but these exogenously dormant cells contain up to 30% more PEA than do zygospores; (3) no considerable differences were revealed between the phospholipid composition of SS and ST (Fig. 1), although ST contain significantly more phospholipids than SS.

However, a comparison in terms of the composition of neutral lipids revealed significant differences between *B. trispora* cells in the endogenous and exogenous dormancy state and between stylo- and zygospores: (1) zygospore neutral lipids virtually consist of reserve lipids (triacylglycerols (TAG)), which account for 92% of the total neutral-lipid content (Fig. 2); (2) ST and SS contain considerably less TAG than do zygospores; (3) ST are characterized by sterol (St) and sterol ester (StE) contents 7 to 12 times higher than in zygospores; and (4) SS contain more free fatty acids than do ST or zygospores.

In contrast to most *Mucorales*, *B. trispora* is carotenogenic. Dormant *B. trispora* cells contain carotenoids in the neutral-lipid fraction, although their content is lower (Table 2) than in vegetative cells (mycelium),

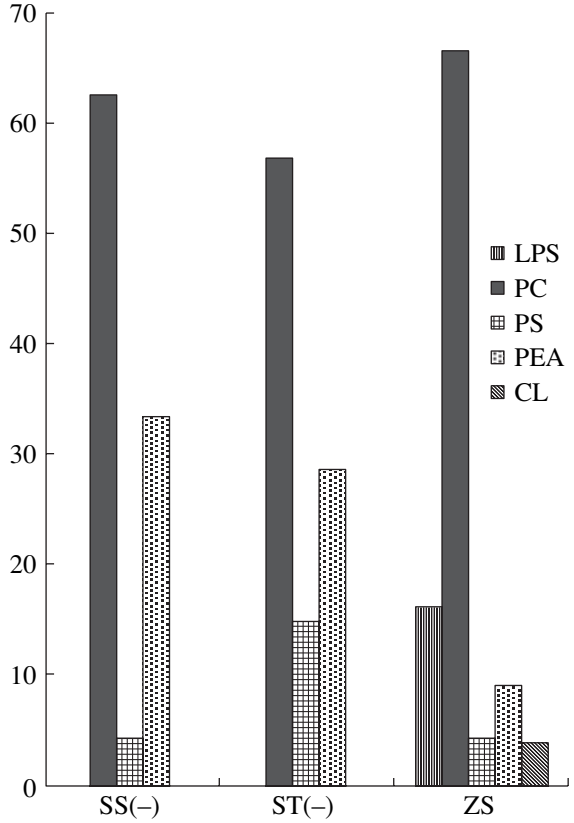

Fig. 1. Phospholipid composition of *B. trispora* (% of total lipids). SS (–), sporangiospores from (–) sporangioles; ST (–), sporangiospores from stylosporangia; ZS, zygospores; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; PEA, phosphatidylethanolamine; CL, cardiolipin.

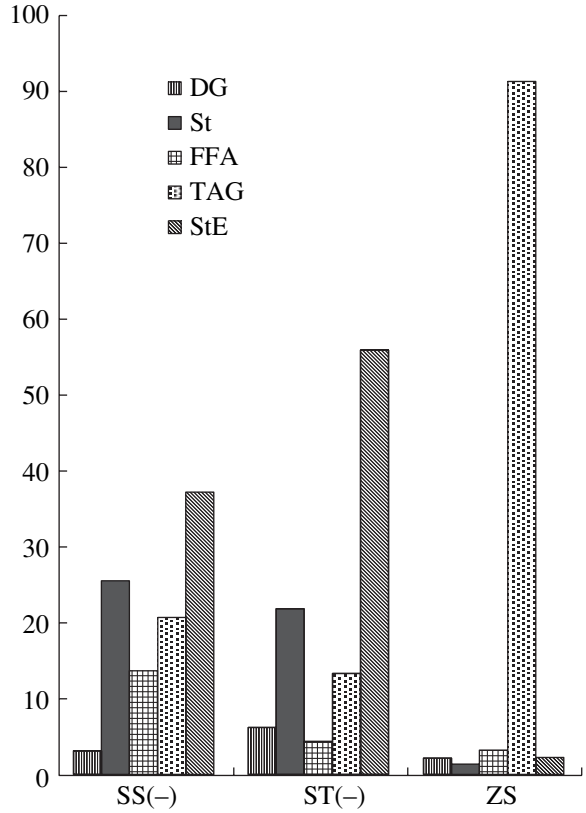

Fig. 2. Neutral-lipid composition of *B. trispora* (% of total lipids). SS (–), sporangiospores from (–) sporangioles; ST (–), sporangiospores from stylosporangia; ZS, zygospores; DG, diacylglycerols; St, sterols; FFA, free fatty acids; TAG, triacylglycerols; StE, sterol esters.

Table 2. Carotenoid composition of sporangio- and zygospores of *B. trispora*

Spore type	Carotenoids, % of dry biomass	Carotenoids, % of total carotenoids		
		γ-carotene	lycopene	β-carotene
SS (–)	0.75	86.3	–	13.7
ST (–)	0.70	92	–	8
Zygospores	0.45	50	33.3	16.7

whose carotene level may reach 6–8% of the dry biomass [12]. It was established that (1) zygospores contain three carotenoid pigments: γ-carotene, lycopene, and β-carotene; (2) sporangiospores of both types lack lycopene; and (3) γ-carotene dominates the carotenoids of all types of dormant *B. trispora* cells.

Based on our studies on the fatty acid composition of individual lipid classes in exogenously and endogenously dormant *B. trispora* cells, we revealed the following patterns (Table 3): (1) the phospholipids of zygospores contain 3–4 times less $C_{18:2}$ than do the phospholipids of ST and SS (this acid prevails in the glyco- and neutral lipid fractions); a peculiarity of the fatty acid composition of zygospores (in contrast to spores) is the predominance of the $C_{16:0}$ acid, which results in a low desaturation degree of the phospholipids; (2) zygospores lack long-chain fatty acids (C_{20}–C_{24}); (3) the phospholipids of zygospores are more saturated than those of ST and SS; and (4) ST glycolipids exhibit low $C_{18:2}$ levels.

No differences in cytosol carbohydrate were detected between *B. trispora* sporangiospores and zygospores. It was shown that trehalose is the predominant carbohydrate in all types of dormant cells and that ST contain a comparatively low amount of glucose (Table 4).

DISCUSSION

Based on our studies with the three types of dormant cells of *B. trispora*, we established for the first time the biochemical criteria that enable us to unambiguously distinguish between endogenous and exogenous dormancy. These criteria are based on the following differences in the carbohydrate composition. (1) Endogenous dormancy (zygospores) is characterized by a high lipid content. The lipids are typically dominated by triacylglycerols and phosphatidylcholine. The predominant acid in zygospore phospholipids is $C_{16:0}$, not $C_{18:2}$, and they lack long-chain fatty acids. Zygospore carotenoids contain a maximum lycopene amount (30–33% of the total carotenoids). (2) Cells in the state of exogenous dormancy (ST and SS) contain significantly less lipids than zygospores. More PEA occurs in their phospholipids as compared to the phospholipids of zygospores, and the neutral-lipid fraction contains considerably more free fatty acids, St, and StE. Linoleic acid dominates the glycolipids and phospholipids. (3) The desaturation degree of neutral lipids/glycolipids and phospholipids in zygospore lipids is higher and lower, respectively, than in sporangiospore lipids. This pattern is particularly manifest in the phospholipid fraction of zygospores, whose desaturation degree is 2–2.5 times lower than that of sporangiospores. (4) Zygospores do not differ from SS and ST in respect to the cytosol carbohydrate composition.

The above differences in the lipid composition suggest that endogenous dormancy secures a more prolonged cell viability period than exogenous dormancy. This suggestion is supported by the data showing that phosphatidylcholine, the most oxidation-resistant phospholipid, is the predominant phospholipid species in zygospores. In addition, zygospores contain very low amounts of PEA, a phospholipid peculiar to actively growing cells. Endogenously dormant *B. trispora* cells contain much lycopene (up to 33% of the total carotenoid fraction), which possesses maximum antioxidant activity among natural antioxidants [13]. Using a microscope, we showed that zygospore neutral lipids are located in specific compartments, which prevents their oxidation, in addition to the effects of antioxidants. These data provide an explanation for the high resistance of zygospores (in contrast to exogenously dormant cells) to environmental factors. Moreover, neutral lipids in TAG form constitute the main lipid fraction in zygospores, and they represent a more energy-rich stock of vital fuel in comparison to carbo-

Table 3a. Fatty acid composition of the phospholipids of spores and zygospores of *B. trispora*

Spore type	Fatty acids, % of total																		DD
	$C_{14:0}$	$C_{14:1}$	$C_{14:2}$	$C_{15:0}$	$C_{15:1}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$	$C_{21:0}$	$C_{22:0}$	$C_{22:1}$	$C_{23:0}$	
SS (–)	1.8	Tr	Tr	5.9	Tr	7.3	5.4	4.3	Tr	1.4	15.1	49.9	Tr	Tr	1.4	1.9	1.4	4.1	1.22
ST (–)	Tr	Tr	Tr	Tr	Tr	7.5	3.6	3.0	Tr	6.0	17.9	44.8	Tr	Tr	Tr	Tr	6.0	11.2	1.17
Zygospores	0.7	Tr	Tr	2.8	Tr	33.5	6.9	17.3	2.8	8.3	15.6	12.1	Tr	–	–	–	–	–	0.50

Note: Here and in tables 3b and 3c, "DD" means "desaturation degree" and "Tr" means "traces".

Table 3b. Fatty acid composition of the neutral lipids of spores and zygospores of *B. trispora*

Spore type	Fatty acids, % of total																DD
	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$	$C_{21:0}$	$C_{22:0}$	$C_{22:1}$	$C_{23:0}$	
SS (–)	Tr	Tr	Tr	31.6	2.8	3.4	Tr	15.8	18.0	24.8	1.8	Tr	1.8	Tr	Tr	Tr	0.76
ST (–)	Tr	Tr	Tr	26.6	3.7	1.7	Tr	15.5	16.5	17.3	4.7	Tr	Tr	7.0	4.2	3.0	0.73
Zygo-spores	Tr	0.6	–	27.0	9.4	Tr	–	4.7	21.2	31.6	5.5	–	–	–	–	–	1.10

Table 3c. Fatty acid composition of the glycolipids of spores and zygospores of *B. trispora*

Spore type	Fatty acids, % of total																DD
	$C_{14:0}$	$C_{14:1}$	$C_{14:2}$	$C_{15:0}$	$C_{15:1}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$	$C_{21:0}$	$C_{22:0}$	$C_{22:1}$	
SS (–)	Tr	Tr	Tr	Tr	Tr	14.0	6.2	31.7	Tr	3.9	9.6	26.2	Tr	2.8	2.8	Tr	2.8	0.74
ST (–)	Tr	Tr	Tr	Tr	Tr	15.1	5.3	33.4	Tr	4.2	15.8	4.2	Tr	3.2	Tr	Tr	Tr	0.33
Zygo-spores	1.8	–	–	0.2	–	22.7	5.4	5.3	0.9	12.6	20.6	26.5	4.0	–	–	–	–	0.92

hydrates. From Table 3, it is evident that sporangiospores and zygospores also differ in fatty acid composition, particularly of the phospholipid fraction. It was established for the first time that $C_{16:0}$ is the predominant fatty acid in the *B. trispora* phospholipids, i.e., that zygospores significantly differ from the mycelium, whose fatty acids are dominated by the unsaturated oleic and linoleic acids [2]. Conversely, the fatty acid composition of sporangiospores is sufficiently similar to that of the mycelium (Table 3a). Interestingly, $C_{16:0}$ is more resistant to oxidation than linoleic acid, which prevails in the membranes of actively dividing fungal cells (in the mycelium). Lysolipids (specifically LPC) are also characteristic of endogenously dormant cells, indicative of significant conformational changes in the zygote membranes.

The data obtained enable us to provide a novel interpretation for the sporangiospore capacity to quickly discontinue exogenous dormancy. Apart from the presence of carbohydrates (a mobile, readily utilizable energy source), sporangiospores are characterized by high levels of St and free fatty acids, which are currently regarded as germination triggers [14]. Free fatty acids are known to control the intracellular cAMP level and to coordinate some of the sporogenesis stages, including spore initiation in *Mucorales* [15]. Zygospores virtually lack the aforementioned neutral lipids (free fatty acids and St). It seems likely that either zygospore germination is controlled by other compounds or, which is more probable, free fatty acid and sterol synthesis only occurs in response to a cytoplasmic signal.

The results reported in this work provide grounds for a suggestion concerning the systematic position of *B. trispora*. The phospholipids of zygospores contain more saturated lipids than the phospholipids of SS and ST. The zygospore phospholipids of *B. trispora* differ from those of other *Mucorales* representatives, such as, e.g., *Absidia coerulea* [16], which contains highly unsaturated lipids. In addition, the fatty acids of the mycelium are dominated by the $C_{18:2}$ acid, not by the $C_{18:1}$ acid typical of *Mucorales*. The cell walls of the hyphae of the (–) strain of *B. trispora* contain 4 times more neutral sugars than those of *Cunninghamella japonica*, a typical representative of *Mucorales* [17]. These data support the doubts we expressed earlier concerning the taxonomic position of *B. trispora* within the family *Choanephoraceae* and our hypothesis that *B. trispora* may be an intermediate form between *Mucorales* and *Ascomycetales* [2].

Table 4. Carbohydrate composition of sporangio- and zygospores of *B. trispora*

Spore type	Carbohydrates, % of dry biomass	Carbohydrates, % of total	
		glucose	trehalose
ST (–)	4.6	18.2	81.8
SS (–)	3.1	28.8	71.2
Zygospores	3.8	23.7	76.3

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