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EXPERIMENTAL ARTICLES

Changes in the Lipid Composition of *Mucor hiemalis* Sporangiospores Related to the Age of the Spore-Forming Culture

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Abstract—Analysis of sporangiospore lipids of the fungus *Mucor hiemalis* F-1156 showed that alterations occur in the content of fatty acids and individual classes of lipids during long-term cultivation (for about 20 days). The changes in the lipid composition related to the age of the spore-forming mycelium suggest an important role of sporangiospore lipids in spore germination and in further development of the spherical cells formed in this processes. The *M. hiemalis* F-1156 sporangiospores with a lipid pool exhausted during long-term cultivation can give rise to both mycelial and yeastlike growth.

Key words: *Mucor*, sporangiospores, lipids, fatty acids.

Like carbohydrates, lipids of the fungal spores are known to serve as a reserve of energy and of structural material utilized by cells at the stage of germination [1]. Lipids are also involved in regulation of biochemical processes, and the cell growth strategy depends on the lipid content and composition [2].

The data available in the literature suggest that the *M. hiemalis* is a monomorphic fungus, i.e., it is unable to display dimorphic growth [3]. While studying the dimorphism of mucorous fungi under aerobic conditions [4–6], we noticed the ability of the *Mucor hiemalis* F-1156 strain to grow during submerged cultivation both in the mycelial and yeastlike form if the inoculum contained sporangiospores from a culture grown on wheat bran. When a fungal culture grown on wort agar served as the inoculum, some strains of this species were unable to display dimorphic growth in liquid medium.

In addition, during submerged cultivation, the fungal ability to develop arthrospores and budding cells was found to increase with the time of the culture growth. In a young culture, sporangiospores gave rise only to mycelial growth. According to data available in the literature, the spore ability to germinate depends on spore age and humidity [7–9]. In addition, alterations were shown to occur in sporangiospore lipid composition during cultivation [10].

We hypothesized that the manifistation of *M. hiemalis* capacity for dimorphism is related to the state of sporangiospores contained in the inoculum, specifically, to the changes in the composition of their structural and reserve lipids during long-term cultivation.

In this work, we studied the qualitative and quantitative composition of fatty acids, as well as of neutral and polar lipids, in sporangiospores of *M. hiemalis* F-1156 cultures of various age. We aimed at clarifying the relationships between changes in the lipid composition of the fungus and its ability to display dimorphic growth.

MATERIALS AND METHODS

A culture of the fungus *Mucor hiemalis* F-1156 was obtained from the All-Russia Collection of Microorganisms (VKM), Russian Academy of Sciences. Fungi were grown for 3, 7, 10, 14, and 20 days in 2-l flasks containing 40 g of wheat bran (70% humidity). After completing the cultivation, sporangiospores were washed off with water from the solid phase surface and sedimented by centrifugation.

Lipids were extracted from sporangiospores by the Folch method [11], and their amount was determined gravimetrically.

Germination of the spores contained in young and old cultures (3- and 20-day-old, respectively) was studied using liquid medium the composition of which was described previously [6]. Flasks containing 50 ml of medium were inoculated with a spore suspension (1.5×10^7 spores per flask). Nongerminated spores were counted on a hemocytometer after one, two, and three days of cultivation.

Fatty acid methyl esters were obtained by methanolysis at 80°C for 90 min in a mixture of methanol and acetyl chloride; then, they were extracted with hexane and analyzed by gas-liquid chromatography on a model 3700 chromatograph (Russia) equipped with a column containing 17% diethylene glycol succinate (DEGS) on

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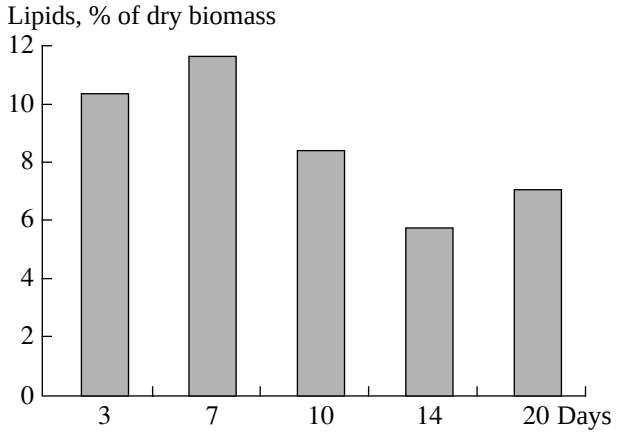

Content of total lipids in spores of *M. hiemalis* F-1156 as dependent on the age of the spore-forming culture.

Chromosorb W; the carrier gas (helium) flow rate was 40 ml/min; the temperature was 170°C.

Lipid class composition was studied by thin-layer chromatography on Kieselgel 60 F_{254} plates (Merk, Germany). Neutral lipids were determined in the hexane–diethyl ether–acetic acid (80 : 20 : 1, v/v system). Two-dimensional chromatography of polar lipids was conducted in the following solvent systems: chloroform–methanol–28% ammonium hydroxide (65 : 25 : 5, v/v) and chloroform–acetone–methanol–acetic acid–water (6 : 8 : 2 : 2 : 1, v/v) for the first and second dimensions, respectively. The plates were developed with either a 10% solution of phosphomolybdic acid in methanol or sulfuric acid.

Lipids were identified by comparison with R_f values of standards and using qualitative reactions with the following reagents: ninhydrin (for nitrogen-containing lipids with a free amino group), α-naphthol (for glycolipids), Dragendorff reagent (for choline-containing lipids), Vas'kovskii reagent (for phospholipids), and a 1 : 1 mixture of sulfuric and acetic acids (for free and esterified sterols) [12]. Quantitative determination of some classes was performed densitometerically, using a MustekA3EP scanning device and the Rastr Pro software.

RESULTS AND DISCUSSION

Young spores of the *M. hiemalis* F-1156 culture were darker than mature ones (7–14 days), which suggests that the content of melanins and the level of metabolic activity were higher in young spores [13].

During fungal growth on bran, the content of lipids in spores increased by the seventh day of growth (11.53%) and then decreased to grow up again at the late stage (20 days, figure). Partial spore dehydration in old cultures (20 days) and an increase in the relative content of lipids may account for the latter observation. Note that the content of fatty acids and individual classes of lipids also changed.

Table 1 shows the composition and relative content of fatty acids in total lipids of sporangiospores. The high content of unsaturated fatty acids and, in particular, an extremely high level of γ-linolenic acid, was determined in the spore lipids, which is typical of the representatives of many species of the order *Mucorales*. At the beginning of sporangiospore development (in the three-day culture), mostly saturated fatty acids, specifically, palmitic and stearic acids, were determined in lipids. During further growth, their level decreased (by the tenth day), while the portion of linoleic and γ-linolenic acids increased simultaneously, which led to a higher degree of total lipid unsaturation. During long-term culture growth, the content of γ-linolenic acid decreased, although remained at a high level, whereas the portion of saturated acids somewhat increased again. These changes in the content of fatty acids may affect the membranes, their stability and functional activity, and, ultimately, spore viability.

The composition of lipid classes differed in sporangiospores of different age (Table 2).

The absolute amount of sterol ester fraction and membrane lipids, including free sterols, polar lipids, and quinones, attained its maximum by the seventh–tenth days and decreased during further fungal growth, although the percentage of free sterols in total lipids remained relatively constant. The levels of other acyl-

Table 1. Fatty acid composition of total lipids of sporangiospores in *M. hiemalis* F-1156 cultures of different age

Spore age, days	Fatty acids % of total								
	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	The degree of unsaturation, Δ/100 mol
3	1.8	0.7	21.1	3.8	6.1	20.9	25.4	20.2	136.1
7	1.9	0.9	15.3	2.8	2.4	14.6	28.4	33.7	175.1
10	1.5	0.9	12.8	2.6	1.1	14.8	32.7	33.6	183.6
14	2.1	0.9	16.2	3.1	3.9	16.3	27.7	30.5	164.9
20	1.5	0.9	15.7	2.5	2.8	17.0	31.1	28.5	168.2

Table 2. Composition of classes of total lipids of sporangiospores in *M. hiemalis* F-1156 cultures of different age

Culture age, days	Lipid content	PL	DAG	Free sterols	Alcohols and methyl sterols	FFA	Quinones	TAG	SE
3	A	22.50	1.58	8.33	3.86	13.05	2.14	32.16	16.38
	B	23.02	1.62	8.18	3.95	13.35	2.19	32.90	16.76
7	A	23.90	3.08	12.12	4.45	4.42	5.07	18.34	28.62
	B	27.56	3.55	13.97	5.13	5.10	5.85	21.15	33.00
10	A	30.11	4.32	13.88	2.86	2.89	3.23	15.13	27.52
	B	24.99	3.58	11.51	2.37	2.39	2.68	12.54	22.81
14	A	20.22	7.17	12.39	3.46	3.72	5.13	25.88	22.03
	B	11.34	4.02	6.95	1.94	2.07	2.88	14.52	12.36
20	A	23.55	7.78	11.74	3.34	6.72	4.26	27.41	15.19
	B	16.20	5.35	8.08	2.80	4.62	2.93	18.85	10.45

Note: A, % of total lipids; B, mg/g of dry spore biomass. PL, polar lipids; DAG, diacylglycerols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters.

containing fractions—triacylglycerols (TAG) and free fatty acids (FFA)—were the highest in the young-culture spores (three days) and decreased by the fourteenth day synchronously with an increase in the level of diacylglycerols (DAG). By the twentieth day, the *M. hiemalis* F-1156 spores contained the lowest amount of esterified and free sterols (a paper on the variation of the content of individual sterols in sporangiospores of the culture under study has been submitted to *Mikrobiologiya*). A reduced portion of reserve lipids may seriously affect spore viability and their capacity to germinate into mycelium.

Table 3 shows the composition of individual classes of lipids. The major phospholipids (PL) were phosphatidylcholine (PC), phosphatidylethanolamine (PEA), cardiolipin (CL), and phosphatidylserine (PS). In addition, phosphatidic acid (PA) and an unidentified phospholipid (R_f 0.61 in system 1) were determined in small amounts. Glycolipids (GL) included cerebrosides with normal fatty acids and hydroxy-acids, glycolipid-1 containing a free amino group (R_f 0.04 in the system 1), and two other glycolipids, GL-2 (R_f 0.24) and GL-3 (R_f 0.70).

In young spores, the content of structural phospholipids was the highest: that of PEA, in a three-day culture, and that of CL, PS, and PA, in the seven-day culture. In more mature spores, the content of these compounds was noticeably reduced. PC accumulated up to the tenth day, and then decreased during further cultivation. The PEA/PC ratio was directly correlated with the intensity of metabolism [14]. This ratio was high in young spores, near 1, and decreased to 0.4 with spore maturation. An increase in the PEA/PC ratio to 0.9 after ten days of culture growth was presumably caused by the utilization of the reserve PC pool in sporangiospores of an aging culture. These spores (with high PEA and low PC content) lost to a great extent the ability to germinate. A decrease in the content of CL, an important component of the mitochondrial membranes, suggests changes in the structural organization of the latter and in sporangiospore functional activity after seven–ten days of growth.

Both the relative and absolute content of glycolipids, specifically, GL-1, GL-3, and total cerebrosides, increased with the time of *M.hiemalis* F-1156 cultivation. In old spores, their content attained the maximum. The PL/GL ratio, which was the highest in young spores and the least in the old ones, suggests that phospholipid synthesis decreased, whereas their mobilization increased. This confirms the structural function of glycolipids [15], as well as their possible involvement in cell differentiation [16–18], which is a widely accepted notion.

The prominent age-dependent changes in sporangiospore lipid composition may be related to the state of mycelium, on which they develop, as well as to the environmental factors of their habitat. Since sporangiospores are not so much surviving as propagating structures, their metabolism is more liable, less inhibited than that of zygospores, and dependent to a greater extent on the cultivation conditions, humidity in particular.

Fungi of the genus *Mucor* belong to a group of mesophiles with respect to the water content in the environment, and they are fairly sensitive to the humidity of the nutrient substrate [8, 19, 20]. During long-term fungal growth on bran, mycelial growth and spore maturation proceeded against the background of changes in the environment, namely, the substrate humidity decreased from 70% at the moment of inoculation to 63% on the twentieth day of cultivation. These conditions promoted partial sporangiospore dehydration.

Long-term cultivation of the *M. hiemalis* 1156 on bran affected sporangiospore ability to germinate. In distilled water, sporangiospores of *M. hiemalis* F-1156 failed to swell, and their germination required the pres-

Table 3. Content and composition of individual fractions of polar lipids in sporangiospores of *M. hiemalis* F-1156 cultures of different age

Culture age, days	Lipid content	GL-1*	PS	PA	GL-2	PC	PEA	CL	Cer 1	Cer 2	Unident. PL	GL-3	PEA/PC	PL/GL
3	A	0.96	8.50	1.60	5.35	30.39	27.33	15.91	2.96	traces	5.00	1.99	0.90	8.47
	B	0.22	1.96	0.37	1.23	6.99	6.29	3.66	0.68		1.15	0.46		
	C	2.16	19.11	3.60	12.03	68.35	61.45	35.77	6.66		11.24	4.48		
7	A	0.44	19.00	3.74	2.22	31.15	20.81	14.69	4.90	0.59	0.84	1.62	0.67	9.24
	B	0.12	5.24	1.03	0.61	8.59	5.74	4.05	1.35	0.16	0.23	0.45		
	C	1.05	45.49	8.94	5.30	74.57	49.83	35.16	11.72	1.39	2.00	3.91		
10	A	1.60	18.69	1.98	4.95	39.42	15.21	7.49	5.14	traces	2.65	2.85	0.39	5.87
	B	0.40	4.66	0.49	1.24	9.88	3.80	1.87	1.28		0.66	0.71		
	C	4.82	56.28	5.96	14.90	118.69	45.80	22.55	15.48		7.98	8.58		
14	A	6.58	16.27	3.59	1.91	23.11	20.46	13.72	10.73	0.04	0.38	3.21	0.89	3.45
	B	0.75	1.85	0.41	0.22	2.62	2.32	1.56	1.22	0.01	0.04	0.36		
	C	13.37	32.87	7.30	3.92	46.70	41.35	27.81	21.75	0.18	0.71	6.42		
20	A	14.70	8.06	3.54	2.14	22.39	20.28	9.00	11.71	traces	3.03	5.55	0.90	1.93
	B	2.38	1.31	0.57	0.35	3.63	3.29	1.46	1.89		0.49	0.89		
	C	34.59	19.04	8.28	5.09	52.76	47.82	21.22	27.47		7.12	12.94		

Note: A, % of total polar lipids; B, mg/g of dry spore biomass; C, mg/g of total lipids. GL, glycolipids; PS, phosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; CL, cardiolipin; Cer, cerebrosides; PL, pospholipids.
* Glycolipid with a free amino group.

ence of nitrogen and carbon sources (urea and glucose, respectively). In a liquid nutrient medium, spore swelling in a young culture (3-day) was observed as soon as 15 min after inoculation and nongerminated spores were absent from the culture liquid by the end of the first day of growth. In old cultures (20 days), spore germination was much delayed under the same conditions, and the portion of nongerminated spores remained high (Table 4). This evidence suggests that the function of *M. hiemalis* F-1156 sporangiospores is to distribute the fungus rather than to promote its prolonged survival. Nevertheless, the heterogeneity of the spore population, which is characteristic of dormant surviving forms, may account for a delay in sporangiospore germination and lead to nonsimultaneous exit of spores from the state of dormancy.

Table 4. Content of nongerminated spores after inoculation of liquid medium with spores from 20-day-old culture of *M.hiemalis* F-1156

Cultivation time, days	Nongerminated spores, % of the inoculum
0	100
1	75
2	36
3	30

Apart from many other factors, the density of inoculum is known to have an effect on spore germination; in the thick suspensions, only single spores germinate. Mass spore germination in the absence of sufficient nutritive substances obviously leads to negative consequences for the total population [10, 21]. However, in our experiments, these factors were of little importance, because a low-density inoculum was used and the content of carbon and nitrogen sources in the medium was high. Long-term cultivation resulted in reduced substrate humidity and caused changes in the spore lipid composition, which most likely affected the spore ability to germinate.

Thus, during long-term cultivation of the fungus *M. hiemalis* F-1156 on bran, alterations occurred in the lipid content and composition of sporangiospores, which have an effect on the survival strategy. The spores of 7- to 10-day-old cultures have an optimal composition of fatty acids and other lipids necessary for maintaining a high level of viability. At a lower level of fatty acid unsaturation, a decreased content of reserve lipids, PC, PEA, PS, and CL, and an increased portion of GL, characteristic of old spores, the general energy potential of the cell is reduced. As a result, spore viability and germination capacity are affected, as well as further development of the spherical cells formed from the spores [4–6]. In *M. hiemalis* F-1156, viable sporangiospores with a lipid pool exhausted during

long-term cultivation give rise to both mycelial and yeastlike growth.

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