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EXPERIMENTAL ARTICLES

Lipid Composition of the Yeastlike and Mycelial *Mucor hiemalis* Cells Grown in the Presence of 4-Chloroaniline

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Abstract—The fungus *Mucor hiemalis* F-1156, which is commonly thought to be monomorphic, produced two types of cells, yeastlike and mycelial, during growth in a medium containing 4-chloroaniline. Among the polar lipids of yeastlike cells, diphosphatidylglycerol was dominant, while phosphatidylcholine and phosphatidylethanolamine were present in minor amounts. Conversely, mycelial cells mainly contained phosphatidylcholine and phosphatidylethanolamine, whereas the content of diphosphatidylglycerol was low. The neutral lipids of yeastlike cells were dominated by diacylglycerides, sterols, and fatty acids. The content of triacylglycerides and sterol esters was low. Yeastlike cells contained higher amounts of saturated fatty acids and lower amounts of unsaturated fatty acids than the mycelium. The content of stearic acid in the fatty acids of the mycelium grown in the presence of 4-chloroaniline was as high as 25.3–29.9%.

Key words: dimorphism, *Mucor*, mycelial fungi, lipids, 4-chloroaniline

Under aerobic conditions, the dimorphism of fungi of the genus *Mucor* (i.e., their ability to grow as yeastlike and mycelial cells) is stimulated by the high concentrations of glucose [1], by chloroanilines [2, 3], and by low pH values of the cultivation medium [3]. Two strains of the species *Mucor hiemalis* also exhibited dimorphic growth [2], although earlier this species was believed to be strictly mycelial [4].

Chloroanilines are toxic compounds, which inhibit oxidative phosphorylation and microbial growth. As a membranotropic substance, 4-chloroaniline also affects the activity of membrane-bound enzymes involved in lipid biosynthesis. According to data available in the literature, the halogen derivatives of certain organic compounds reduce membrane potential and degrade microbial membranes [5]. Azoles affect membrane fluidity and modify the activity of plasma membrane enzymes involved in the metabolism of fatty acids, sterols, and chitin [6].

As shown earlier, the yeastlike and mycelial *Mucor lusitanicus* cells grown in the presence of 4-chloroaniline differ in their fatty acid composition [3]. The study of the lipid composition of cells in relation to their dimorphism is of great interest, since the inhibition of the synthesis of fatty acids can influence the morphogenesis of fungi, as shown, for instance, in *Mucor racemosus* [7].

In most studies devoted to fungal dimorphism, yeastlike and mycelial forms were grown separately under different temperatures, redox conditions, etc., which presumably could affect the results of these studies. Ghannoum *et al.* first reported that the yeastlike and mycelial cells of *Candida albicans* can be obtained in one culture [8]. Such an approach allows a more precise evaluation of the differences associated with the composition of fatty acids and lipids.

The aim of the present work was to study the composition of the polar and neutral lipids of yeastlike and mycelial *M. hiemalis* F-1156 cells grown in one culture in the presence of 4-chloroaniline.

MATERIALS AND METHODS

The strain *Mucor hiemalis* F-1156 used in this study was obtained from the All-Russia Collection of Microorganisms (VKM), Russian Academy of Sciences.

The suspension of sporangiospores for inoculation was prepared by washing them off of the culture grown on bran at 28°C for 7 days. The inoculum contained 10^7 sporangiospores. The fungus was grown in the medium described earlier [2] in the presence of 150 μg/ml 4-chloroaniline. Cultivation was performed in 250-ml flasks with 50 ml of the medium at 28°C on a shaker (130 rpm). Samples for microscopic and additional studies were taken after 1, 2, 3, 4, 9, and 14 days of cultivation. Mycelium was separated from the culture liquid by filtration through a nylon filter. Yeastlike cells, which were retained in the culture liquid filtrate, were collected by centrifugation. The biomass of mycelium was determined gravimetrically after drying it to a constant weight. The number of yeastlike cells in the culture liquid was determined using a hemocytometer.

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Lipids were extracted according to the description of Folch *et al.* [9]. Lipid composition was studied by thin layer chromatography on Kieselgel 60 F-254 plates (Merck, Germany) in a hexane–diethyl ether–acetic acid (80 : 20 : 1) system (neutral lipids), or in a chloroform–methanol–25% ammonia (65 : 25 : 4) system (polar lipids). Lipid spots were visualized by spraying the developed plates with 10% phosphomolybdic acid in ethanol. Lipids were identified by comparing their R_f values with those available in the literature and those determined experimentally using the authentic samples. In addition, lipids were identified in qualitative reactions with ninhydrin (a test for nitrogen-containing lipids with unsubstituted amino groups), Dragendorff reagent (a test for choline-containing lipids), α-naphthol (a test for glycolipids), and a 1 : 1 mixture of sulfuric and acetic acids (a test for sterols and sterol esters) [10]. The densitometry of plates was carried out in a Chromoscan-3 densitometer (Joyce Loebl, United Kingdom), and the content of particular lipids was expressed as a percentage of the total. The fatty acid composition of lipids was determined by gas–liquid chromatography after acid methanolysis [3].

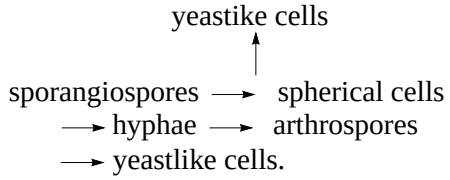
RESULTS AND DISCUSSION

The 1-day-old culture of *M. hiemalis* F-1156 grown in a medium with 4-chloroaniline did not contain budding cells if the spores used for inoculation were washed off from the 4-day culture. If spores from 7-day and older cultures were used for inoculation, *M. hiemalis* F-1156 grew as yeastlike cells when cultivated for one day and longer. These data suggest that the growth strategy of fungi is determined by the age of the spores used for inoculation.

The data presented in Figs. 1 and 2 show that 150 μg/ml of 4-chloroaniline added to the cultivation medium suspension promoted the development of mycelium at the early stages of growth but did not influence the formation of spherical cells. As a result, after 1 day of growth, the culture represented a collection of spherical cells in suspension, some of which contained a mono- or multipolar arrangement of short germ tubes. Within one day, the number of cells with germ tubes increased, and the culture was found to contain a number of budding cells and short chains of 2–4 cells, as well as normal mycelium with intercalar and apical arthrospores. In the 4-day culture, the biomass of mycelium reached a maximum and the population of yeastlike cells became very polymorphic. It included spherical cells without buds, mono- and multipolar budding cells containing up to six buds, and short chains composed of two to eight cells. Since budding yeastlike cells were absent in the 1-day-old culture, we assumed that such cells appear from the arthrospores detached from the hyphae [2]. The possibility, however, cannot be excluded that spherical cells, which are formed from sporangiospores, may also give rise to yeastlike cells.

After 9 days of cultivation, the nonmycelial fraction of the culture mainly contained chains of five to seven budding cells. In addition, long chains of arthrospores (up to 20 in number) were observed. Over longer periods of cultivation, the number of yeastlike cells increased and the arthrospore chains began to break up into individual cells undergoing budding. In the 14-day culture, arthrospore chains were absent and the mycelial biomass decreased, probably due to the detachment of arthrospores from the hyphae and/or hyphae lysis. It should be noted that spherical cells were observed in the fungal culture throughout the cultivation period, albeit in different amounts.

Thus, the aerobic culture of *M. hiemalis* F-1156 develops in the presence of 4-chloroaniline according to the following scheme:



Analysis of the fatty acid compositions of yeastlike and mycelial cells showed that they are different (Table 1). Recall that the 1-day-old *M. hiemalis* F-1156 culture contained only nonbudding spherical cells, which cannot be considered genuine yeastlike cells. In the 2-day and older yeastlike cells, the fraction of saturated fatty acids was greater than in the mycelium and gradually increased in the process of cultivation. As opposed to the mycelium, the yeastlike cells contained more palmitoleic acid and less oleic, linoleic, and γ-linolenic acids. As a result, the coefficient of lipid unsaturation for yeastlike cells was greater than that for mycelium throughout the cultivation period. Similar data were reported for *C. albicans*, yeastlike cells of which were found to contain less linoleic and linolenic acids than the mycelium [8]. It is known that desaturation processes in fungi are very sensitive to respiration inhibitors. Data on the relationship between cell morphology and the intensity of respiration are contradictory. For instance, the yeastlike mutant of *Mucor* was found to be deficient in respiration [11]. On the other hand, the yeastlike growth of *Mucor rouxii* was stimulated by phenethyl alcohol but respiration and cytochrome levels did not decrease in this case [12]. According to earlier observations, the content of saturated stearic acid in the 4-day-old *M. hiemalis* mycelium grown in the absence of 4-chloroaniline is low (8.3%) [13]. The high level of saturated fatty acids in the yeastlike and mycelial cells of this species grown in the presence of 4-chloroaniline may be indicative either of its direct action on the membrane structure or of a partial inhibition of desaturating enzymes.

Various desaturation reactions differ in their sensitivity to the halogen derivatives of compounds with a benzene ring. For instance, trifluoperazine (TFP), a calmodulin inhibitor, was found to affect the content of

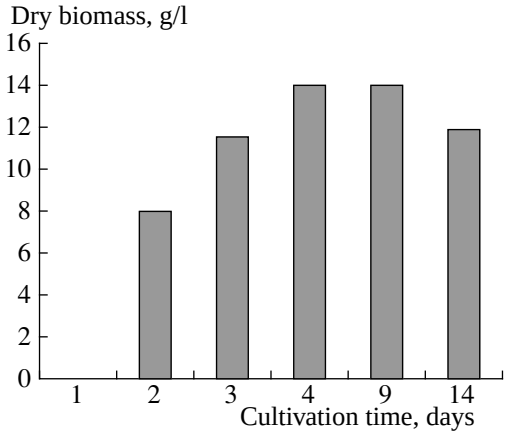


Fig. 1. Accumulation of mycelial biomass in the *M. hiemalis* F-1156 culture grown in the presence of 4-chloroaniline.

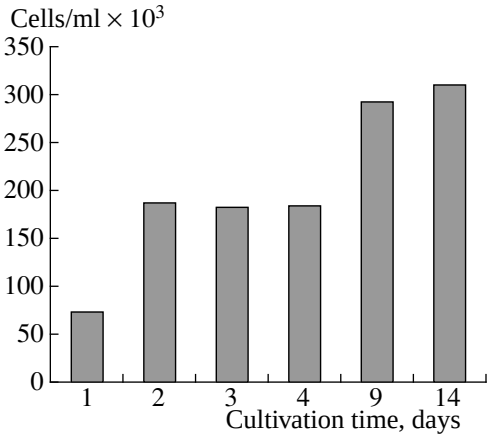


Fig. 2. Accumulation of yeastlike cells in the *M. hiemalis* F-1156 culture grown in the presence of 4-chloroaniline.

particular fatty acids in the lipids of mycelial fungi and to suppress germ tube formation in the germ tube–inducing medium without influencing the yeastlike growth of *Ophiostoma ulmi* [14]. This inhibitor also suppressed the incorporation of fatty acids (FAs) into phosphatidylcholine (PC), phosphatidylethanolamine (PEA), and triacylglycerides (TAGs) in *Mortierella ramanniana*, which led to a decrease in the desaturation rate of linoleic acid. In spite of the decrease in the total content of γ-linolenic acid, the content of this fatty acid (in particular, lipids) virtually did not change, suggesting the existence of at least two pathways of Δ6-desaturation with different sensitivities to TFP [15]. According to data available in the literature, yeastlike and mycelial cells also differ in the composition of neutral and polar lipids.

The small fraction of neutral lipids of *M. hiemalis* F-1156 (Table 2) contained diacylglycerides (DAGs), TAGs, sterols, higher alcohols, free fatty acids (FFAs), alkyl and sterol esters, as well as two compounds (X_1 and X_2) preliminarily identified as naphtho- and benzoquinone, respectively [10].

As mentioned above, the arthrospores that were formed on 2-day-old mycelium remained bound to it. In the 9-day culture, the arthrospore chains were predominantly detached from the mycelium and occurred in the culture liquid along with the yeastlike cells. In the 14-day culture, the arthrospore chains completely fragmented into individual cells. We were unable to separate budding cells from arthrospores at this cultivation stage. This might be the reason for the considerable difference in the lipid composition of the nonmycelial fractions of 9- and 14-day-old cultures.

The fraction of neutral lipids of 9-day and younger yeastlike cells differed from that of mycelium in that they had a higher content of DAGs, sterols, alcohols, free fatty acids, and a lower content of TAGs and sterol esters. The elevated level of sterols and lower levels of TAGs and sterol esters were observed earlier in the yeastlike cells of *C. albicans*, in contrast to the small amount of these substances in the mycelium [8]. The high content of DAGs in yeastlike cells can be associated with the active synthesis of phospholipids in the exponential growth phase. During the course of cultivation (within 9 days), the content of DAGs, sterols, and FFAs decreased, and that of TAGs and sterol esters increased.

In the 9-day and younger mycelial cells, the content of FFAs was reduced, while that of TAGs and sterol esters was elevated. By the 14th day of cultivation, the relative content of polar lipids and FFAs in both yeastlike and mycelial cells rose and that of the TAGs diminished. This was probably associated with the enhanced activity of lipases in senescent cells and the utilization of reserve cell lipids.

Fungal dimorphism can be due to several reasons. As shown in experiments with *C. albicans*, azoles impair the synthesis of ergosterol and cause the accumulation of 14-methylsterol. This affects membrane fluidity and leads to the inhibition of fatty acid desaturation [6]. As a result of a series of regulatory events, the mycelial morphotype transforms into the yeastlike morphotype [16].

The inhibitors of ergosterol synthesis can affect the synthesis of chitin and thereby its content and distribution in yeastlike cells. Impaired chitin synthesis may be responsible for enhanced hyphal branching and swelling of hyphal apices [6].

It was not our aim to study the composition of *M. hiemalis* sterols. According to data presented in Weete's review [17], *M. hiemalis* sterols include cholesterol, ergosterol, and their derivatives. The elevated level of sterols in the yeastlike cells grown in the presence of 4-chloroaniline suggests that they can influence the activity of membrane chitin synthetase.

There is evidence that changes in the composition of membrane phospholipids can affect the activity of fungal chitin synthetase [18, 19]. Therefore, studies of the

Table 1. The fatty acid composition of the yeastlike and mycelial *M. hiemalis* F-1156 cells grown in the presence of 4-chloroaniline

FA, % of total	Yeastlike cells						Mycelial cells					
	1* day	2 days	3 days	4 days	9 days	14 days	1** day	2 days	3 days	4 days	9 days	14 days
C < 14	4.51	1.23	5.05	2.27	1.94	1.43	–	0.84	1.71	1.09	0.75	1.33
C 14 : 0	3.75	3.29	4.32	3.41	3.48	4.40	–	2.53	2.59	2.85	2.67	4.70
C 15 : 0	0.36	0.72	1.06	0.87	0.47	0.63	–	0.54	0.30	0.55	0.59	0.59
C 16 : 0	20.77	24.32	24.95	25.42	22.42	23.17	–	22.07	21.06	21.44	24.71	20.06
C 16 : 1	0.63	1.51	1.33	2.30	1.70	1.84	–	0.36	1.04	1.00	0.88	0.85
C 18 : 0	28.71	29.64	30.31	32.13	31.24	32.92	–	25.33	26.84	26.76	26.86	29.92
C 18 : 1	20.62	21.24	17.37	19.14	18.81	18.76	–	23.63	23.25	23.26	20.52	20.15
C 18 : 2	14.04	12.60	10.84	10.88	14.78	11.33	–	16.46	15.82	15.84	15.94	14.30
C 18 : 3	6.61	5.45	4.77	3.58	5.16	5.52	–	8.24	7.39	7.21	7.08	8.10
Saturated-to-unsaturated FA ratio	0.77	0.69	0.57	0.58	0.70	0.61	–	0.96	0.91	0.92	0.81	0.79

* All yeastlike cells were spherical.
** Mycelium did not grow.

Table 2. The composition of the fraction of neutral lipids of the yeastlike and mycelial *M. hiemalis* F-1156 cells grown in the presence of 4-chloroaniline

Substance, % of total	R_f	Yeastlike cells						Mycelial cells					
		1* day	2 days	3 days	4 days	9 days	14 days	1** day	2 days	3 days	4 days	9 days	14 days
DAGs	0.09	23.43	22.18	20.03	12.58	10.38	8.52	–	6.43	7.87	9.37	6.58	7.02
Sterols	0.15	11.64	12.85	14.85	11.04	8.78	9.06	–	8.54	7.85	8.84	6.65	7.68
Higher alcohols	0.20	5.92	6.23	7.19	6.73	3.91	6.04	–	3.28	3.13	3.19	3.20	2.44
FFAs	0.30	37.94	26.72	18.07	13.92	10.65	12.94	–	14.62	6.58	5.60	4.07	15.23
X_1	0.32	Traces	Traces	Traces	Traces	Traces	Traces	–	Traces	1.82	1.49	1.65	1.25
X_2	0.38	3.05	6.07	4.67	2.90	2.51	3.25	–	1.32	2.65	2.15	2.19	1.25
TAGs	0.60	17.13	20.23	25.52	36.31	43.89	33.13	–	49.36	47.83	47.86	47.46	39.98
Alkyl esters	0.65	Traces	0.68	1.12	Traces	Traces	2.79	–	Traces	Traces	Traces	Traces	Traces
Sterol esters	0.90	0.89	5.04	8.55	16.52	19.88	24.27	–	16.45	22.27	21.50	17.42	25.15

* All yeastlike cells were spherical.
** Mycelium did not grow.

composition of polar lipids, the major membrane constituents, could aid in elucidating their relationship with the dimorphism of *M. hiemalis*.

As can be seen from the data presented in Table 3, the total amount of polar lipids in yeastlike cells was greater than in the mycelium. The polar lipids were represented by diphosphatidylglycerol (diPG), PC, PEA, phosphatidylserine (PS), glycolipids (GLs) with R_f values of 0.00, 0.05, 0.45, and 0.74, and an unidentified phospholipid with $R_f = 0.20$.

Diphosphatidylglycerol was the dominant phospholipid of the yeastlike, and especially of the spherical cells, of *M. hiemalis*. According to some data, the high content of diPG may be indicative of developed membrane organelles, particularly mitochondria, in yeast cells, and of their high respiratory activity [20]. In the process of cultivation, the content of diPG and of the GL with unsubstituted amino acid ($R_f = 0.05$) diminished, while the content of PC and PEA increased. One-day-old yeastlike cells contained GL with $R_f = 0.74$, the level of which decreased to trace amounts in older cells. The GL with $R_f = 0.45$, also observed in trace amounts, gave a weak positive reaction with the Dragendorff reagent, which is an indication of a high unsaturated fatty acid content in this glycolipid [10].

The dominant phospholipids of mycelial cells were PC and PEA, while the GL with $R_f = 0.05$, diPG, PS, and the phospholipid with $R_f = 0.20$ were present in minor amounts. Within 9 days of cultivation in the presence of 4-chloroaniline, the contents of the GLs, PS,

Table 3. The composition of polar lipids of the yeastlike and mycelial *M. hiemalis* F-1156 cells grown in the presence of 4-chloroaniline

Substance, % of total polar lipids	R_f	Yeastlike cells						Mycelial cells					
		1* day	2 days	3 days	4 days	9 days	14 days	1** day	2 days	3 days	4 days	9 days	14 days
GL	0.00	3.08	Traces	Traces	Traces	Traces	Traces	–	Traces	Traces	Traces	Traces	Traces
GL	0.05	18.39	17.23	13.77	9.60	13.19	13.20	–	20.42	19.36	18.74	13.47	26.99
PS	0.13	–	3.67	Traces	Traces	Traces	Traces	–	5.31	3.26	1.50	Traces	2.68
Unidentified lipid	0.20	–	9.18	8.23	Traces	Traces	5.72	–	6.50	8.23	4.09	1.47	4.29
PC	0.27	6.64	12.98	16.34	22.14	25.77	23.47	–	25.18	30.56	29.09	36.54	15.80
PEA	0.34	12.03	19.03	20.61	25.09	28.87	22.74	–	30.04	28.21	29.32	35.03	25.93
diPG	0.41	56.18	37.91	41.05	43.17	32.17	34.87	–	12.55	10.38	17.26	13.49	24.31
GL	0.45	–	–	Traces	Traces	Traces	Traces	–	Traces	Traces	Traces	Traces	Traces
GL	0.74	3.68	Traces	Traces	Traces	Traces	Traces	–	Traces	Traces	Traces	Traces	Traces
Polar lipids, % of all lipids		29.20	26.69	30.04	21.56	22.23	29.92	–	17.60	14.15	13.57	10.96	11.61

* All yeastlike cells were spherical.
** Mycelium did not grow.

and the phospholipid with R_f = 0.20 gradually diminished, and that of the PC rose. In the 14-day culture, the content of mycelial GL and diPG was elevated and that of PC and PEA was lowered, probably due to culture senescence.

Wiebe *et al.* reported that the addition of choline to the growth medium of *Fusarium graminearum* A 3/5 prevents branching and enhances hyphal extension [21]. The role of PC in the normal functioning of chitin synthetase was emphasized by Kodama *et al.*, who found that the phosphoorganic fungicide Kitazin P inhibits chitin synthesis in *Pericularia oryzae* by suppressing the synthesis of PC [19]. The higher content of PC in the *M. hiemalis* F-1156 mycelium than in the yeastlike cells suggests that this phospholipid is involved in morphogenesis.

The biocidal effect of many halogen-substituted aromatic compounds is considered to be related to the inhibition of the plasma membrane ATPase. However, Kuyyakanond and Quesnel (1992) found that one of these compounds, chlorhexidine, does not inhibit the membrane ATPase of *Escherichia coli* even at high concentrations, but does impair the permeability of the plasma membrane. In this case, cells with a higher PEA content were more resistant to chlorhexidine [5]. In our opinion, the high ability of the mycelial cells of *M. hiemalis* F-1156 to grow in the presence of 4-chloroaniline (the biomass accumulated was 14 g/l) is related to the high content of PEA in these cells.

Thus, the lipid profiles of yeastlike and mycelial *M. hiemalis* F-1156 cells are qualitatively similar but quantitatively different. 4-Chloroaniline affects cell membranes and induces changes in the relative content of fatty acids, phospholipids, neutral lipids, and sterols, thereby promoting the yeastlike growth of *M. hiemalis* F-1156. However, the possibility cannot be excluded that the yeastlike growth of fungi may be induced by factors other than 4-chloroaniline.

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