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EXPERIMENTAL ARTICLES

Sporangiospore Lipids of the Mycelial Fungus *Mucor ramannianus* Incapable of Dimorphic Growth

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Abstract—Members of the species *Mucor ramannianus* are believed to be monomorphic. They grow only as a mycelium and are not capable of growth as budding cells, i.e., of dimorphic growth. In our study, we investigated the lipid composition of *M. ramannianus* sporangiospores, which retained the capability of initiating mycelial growth in the course of long-term cultivation of the spore-forming mycelium. It was demonstrated that sporangiospores contained high concentrations of triacylglycerides (TAG) in their reserve lipids and high concentrations of phosphatidylcholine (PC) in their membrane lipids; low concentrations of methylated ergosterol precursors were detected among sterols. On the basis of the data presented, in order to evaluate the potential of mucor fungi for yeastlike growth, it has been suggested to analyze the qualitative and quantitative characteristics of their sporangiospore lipids and to consider the following criteria as the criteria of sporangiospore capacity for giving rise to yeastlike growth upon spore germination: (1) the phosphatidylethanolamine/phosphatidylcholine (PE/PC) ratio; (2) the level of ergosterol and the ratio between the methylated and demethylated sterols; as well as (3) the ratios between phospholipids and glycolipids (PL/GL) and (4) between etherified and free sterols (ES/FS).

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Capacity for dimorphic growth is a common trait of fungi; it is an adaptive reaction to environmental changes. However, many species of mucor fungi vary in their capabilities for yeastlike growth. According to the published data, species which can easily switch to yeastlike growth include *Mucor rouxii, M. racemosus, M. genevensis, M. bacilliformis*, and *M. circinelloides.* Among the members of the species *M. subtilissimus*, some strains are capable of yeastlike growth. Members of the species *M. mucedo, M. miehei, M. pusillus, M. hiemalis*, and *M. ramannianus* [1] are considered monomorphic; they can grow only in the form of a mycelium.

In our previous works, we have demonstrated that some strains of *M. hiemalis* are also capable of yeastlike growth in aerobic conditions under the influence of chloranilines [2, 3] or as a result of long-term cultivation of the spore-forming mycelium on wheat bran [4]. Changes in the character of sporangiospore germination, depending on many factors, can also be attributed to changes in the lipid composition, namely, in the amounts of the principal structural lipids (phospholipids, glycolipids) and reserve lipids (triacylglycerides, sterol esters), as well as to the sterol composition during long-term cultivation of spore-forming cultures [5]. The *M. hiemalis* sporangiospores with low ergosterol content and the lipid pool exhausted during long-term cultivation gave rise to both mycelial and yeastlike growth.

In this work, we studied the lipid composition of *M. ramannianus* sporangiospores, which were able to give rise to mycelial growth after long-term cultivation of spore-forming cultures.

MATERIALS AND METHODS

In our study, we used the strain *Mucor ramannianus* F-530 obtained from VKM, Russian Academy of Sciences. After cultivation for 7, 13, and 20 days in two-liter flasks containing 40 g of wheat bran (humidity 70%), sporangiospores were washed off from the solid phase with water and centrifuged. For the observation of spore germination, we used the liquid medium described in [4].

Sporangiospore lipids were extracted using the Folch method [6]; their amounts were determined gravimetrically.

The lipids were subjected to alkaline hydrolysis in ethanol (5 ml) supplemented with 0.2 ml of the 33% KOH solution at 80°C for 3 h. The unsaponifiable frac-

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Table 1. Total lipid composition of the *M. ramannianus* F-530 sporangiospores

Age of the spore-forming culture, days	Lipids, % of total lipids							Lipids, % of dry biomass
	PL	DAG	Sterols	Methylsterols and alcohols	Free fatty acids	TAG	ES	
7	15.6	7.3	12.9	2.2	13.8	41.5	4.6	6.2
13	18.1	6.7	14.3	1.9	13.4	38.2	7.3	7.6
20	21.1	4.3	13.4	0.9	5.2	45.4	9.0	3.7

tion was extracted with three portions of hexane, washed with distilled water, and then dried with anhydrous sodium sulfate and dissolved in benzene. Identification of lipids and determination of the relative sterol content were performed by gas-liquid chromatography–mass spectrometry (GLC–MS) and gas-liquid chromatography (GLC), as described in [7]. Free fatty acids were extracted with hexane after acidification of the ethanol solution of the saponated lipids with 6 N HCl, evaporated, and subjected to methanolysis in a mixture of methanol and acetyl chloride at 80°C for 1.5 h.

Methyl esters of fatty acids (MEFA) were extracted with hexane and analyzed by GLC in the isothermal mode at 170°C in a Model 3700 chromatograph (Russia), equipped with a column with 17% diethyleneglycol succinate on Chromosorb W, at gas-carrier (helium) consumption of 40 ml/min.

The composition of lipid classes was determined by thin-layer chromatography on TLC plates (Kieselgel 60 F_{254}, Merck, Germany). To determine the neutral lipid composition, the solvent system of hexane : diethyl ether : acetic acid (80 : 20 : 1 by volume) was used. Two-dimensional chromatography of polar lipids (PL) was carried out using the solvent systems of chloroform : methanol : 28% ammonia (65 : 25 : 5 by volume) in the first direction and of chloroform : acetone : methanol : acetic acid : water (6 : 8 : 2 : 2: 1 by volume) in the second. Sulfuric acid or 10% solution of phosphomolybdenic acid in methanol were used as developers.

To identify the lipids present, the qualitative reactions with ninhydrin (for lipids containing free amino groups), α-naphthol (for glycolipids), Dragendorff's reagent (for choline-containing lipids), Vas'kovskii's reagent (for phospholipids), and a mixture of sulfuric and acetic acids (1 : 1, for free and etherified sterols) were carried out, along with the comparison with R_f of internal controls [8]. The quantitative analysis of some lipid classes was performed densitometrically, as described in [5].

RESULTS AND DISCUSSION

The *M. ramannianus* sporangiospores were light pink and swelled after 4 h of incubation in a liquid nutrient medium. At the end of the first day of incubation, the spherical cells obtained from the sporangiospores of the 7, 13, and 20-day cultures germinated only into mycelium; no yeastlike cells developed from spores were observed. The viability of the sporangiospores derived from young and old cultures did not change with age. They gave rise to finely dispersed mycelial growth as close compact microcolonies with thickened vacuolated hyphal fragments and large spherical cells with thin cell walls and granular contents, which could not be identified as arthrospores. These cells were positioned at the apical and intercalary regions of the mycelium and remained linked to it. In *M. ramannianus*, we observed no yeastlike budding cells which can evolve from such cells when separated from the mycelium, as in the case of *M. hiemalis* and *M. lusitanicus*. In comparison with the 7-day cultures, the hyphae developed from the growth tubes during the germination of the sporangiospores of the 13 and 20-day cultures were thicker, and the mycelium was deformed to a greater extent.

The ability of mycelial fungi to accumulate lipids is a genetically determined trait. According to the published data, in the *M. ramannianus* mycelium, their proportion was more than 15% [9]; however, it was low in sporangiospores (Table 1).

On the whole, the lipid content in sporangiospores was stable. The principal reserve lipids were represented by triacylglycerides (TAG) (Table 1). As the cultivation time was extended, the content of free fatty acids changed significantly; their decrease was accompanied by an increase in the percentage of polar lipids (PL) and sterol esters (SE). The level of free sterols and TAG was high and did not vary significantly in the spores of different age. The ratio between etherified and free sterols (ES/FS) was low and relatively stable (0.3, 0.5, and 0.6 for the spores from the 7, 13, and 20-day cultures, respectively). This fact makes *M. ramannianus* different from *M. hiemalis* and *M. lusitanicus*; in the latter organisms, the ES level was higher, and the decrease in the reserve lipid contents (TAG and ES) correlated with the decrease in the spore viability and the induction of yeastlike growth [5, 10].

The fatty acid composition of sporangiospores is shown in Table 2. The *M. ramannianus* lipids, unlike the lipids of *M. hiemalis* and *M. lusitanicus*, contained low concentrations of γ-linolenic acid; however, its level in all the above-mentioned cultures increased during the spore-forming cultivation simultaneously with an increase in the linoleic acid concentration and a

Table 2. Fatty acid composition of the *M. ramannianus* F-530 sporangiospores

Age of the spore-forming culture, days	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{16:2}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	Unsaturation degree, Δ/100
7	1.0	2.1	0.6	29.9	2.9	0.6	7.0	27.2	21.9	5.0	1.7	90.1
13	1.6	2.2	0.6	20.3	3.9	0.8	5.2	25.1	29.8	9.1	1.1	117.5
20	0.5	1.5	0.5	19.8	3.2	1.2	6.0	23.5	29.1	13.2	1.4	126.9

Table 3. Composition of some classes of polar lipids of the *M. ramannianus* F-530 sporangiospores

Age of the spore-forming culture, days	*X*-1	GL*	PS	PA	SL-1	SL-2	PC	PE	CL	C-1	C-2	GL	*X*-2	PE/PC	PL/GL
7	7.7	0.5	4.4	7.5	5.9	7.3	23.7	19.4	5.3	8.9	3.8	3.5	2.1	0.8	2.1
13	4.8	–	0.1	3.6	4.0	9.2	27.5	23.1	5.9	1.8	8.9	6.8	4.3	0.8	2.0
20	–	–	–	6.0	4.7	8.3	41.6	17.8	5.5	3.3	6.2	3.0	3.6	0.4	2.8

* Glycolipid with a free amino group; PS, phosphatidyl serine; PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; CL; cardiolipin; *X*-1 *X*-2, unidentified lipids; C-1, C-2, cerebrosides; SL-1, SL-2, sulfolipids.

decrease in the concentration of palmitic and oleic acids.

Among the polar lipids of the 20-day culture sporangiospores, an increase in the concentration of the principal phospholipid, phosphatidylcholine (PC), and, consequently, a decrease in the PE/PC ratio were noted (Table 3). The high PC level and, accordingly, low values of the PE/PC ratio appear to be reliable criteria for the capacity of spore germination and may indicate their viability [11]. Moreover, *Mucor ramannianus* spores differed from those of *M. hiemalis* and *M. lusitanicus* in that their membrane lipids contained less glycolipids [5, 10]. It is indicative that the phospholipid/glycolipid (PL/GL) ratio did not significantly change, suggesting that the membranes were stable.

The sterol composition of the 20-day culture of *M. ramannianus* is shown in Table 4. Ergosterol was the principal sterol, and, among its precursors, fecosterol was the principal one. The content of methylated precursors of ergosterol, including C-14 methylated triterpene eburicol and 24-methylene-4,14-dimethyl cholesterol, was very low (2%) in comparison with the spores of the 20-day culture of *M. hiemalis*, where their content was as high as 13% [12]. The ratio between methylated and demethylated sterols was 1 : 24 (for comparison, in the old 20-day culture of *M. hiemalis*, this ratio was 2 : 3). It seems likely that the ability of sporangiospores to germinate and develop into a mycelium is associated not so much with the ergosterol content as with the low content of its methylated precursors incapable of maintaining the membrane structure and their normal functioning. Accumulation of 14-methylsterols due to the inhibition of C14-demethylase is known to be incompatible with the structural integrity of membranes [13, 14].

The ergosterol percentage among the total sterols in sporangiospores of the old *M. hiemalis* cultures [12], which give rise to mostly yeastlike and reduced myce-

Table 4. Sporangiospore sterols of the 20-day culture of *M. ramannianus* F-530

Sterols	Relative content (% of the total sterols)
24-methyl-cholesta-5,7,22-trien-3β-ol (ergosterol)	64.1
24-methyl-cholesta-7,22-dien-3β-ol (5-dihydroergosterol)	0.5
24-methyl-cholesta-5,22-dien-3β-ol (7-dihydroergosterol)	7.0
24-methylen-cholest-8-en-3β-ol (fecosterol)	19.0
24-methylene-cholest-7-en-3β-ol (episterol)	5.7
24-methylene-4α-methyl-cholest-8-en-3β-ol (4-methylfecosterol)	1.9
24-methylene-4,14-dimethyl-cholest-5-en-3β-ol	1.3
24-methylene-lanost-8-en-3β-ol (eburicol)	0.6
Demethylated/methylated	25.3

lial growth with enhanced arthrospore formation, was comparable to that in *M. ramannianus* sporangiospores (51.4% and 64.1%, respectively), whereas the percentage of methylated sterols was much higher (40.1% and 3.8%, respectively). The high content of desmethylsterols, including ergosterols, contributed to the maintenance of the structure and normal functioning of the membrane, and correlated with the mycelial growth of fungi [13].

In *M. ramannianus* sporangiospores, the high level of fecosterol, the first of the demethylated derivatives of ergosterol biosynthesis, has captured our attention. Its accumulation points to a partial blocking of the Δ8 ⟶ Δ7 isomerization stage [15]. It was demonstrated that in the *Cryptococcus neoformans* isolates resistant to amphotericin B, the main body of sterols was represented by Δ8-containing compounds, including fecosterol [16]. Fecosterol seems to be capable of fulfilling the function of ergosterol, at least partially. It is possible that its presence promotes the mycelial growth of *M. ramannianus*, impaired as it is.

It is well-known that the ergosterol level may influence the activity of chitin synthetases, integral membrane enzymes responsible for the synthesis of chitin, the main component of fungal cell walls [17, 18]. Impaired expression of the chitin synthetase gene I (*chs*1) in *Neurospora crassa* resulted in the formation of aberrant hyphae [19]. There are indications that increased chitin synthesis under the influence of low concentrations of ergosterol biosynthesis inhibitors resulted in irregular chitin distribution in the cell wall (especially in the swollen parts of hyphae and in the septal segments) of the mycelial fungi *Ustilago maydis*, *Penicillium italica*, *Aspergillus fumigatus*, and *Candida albicans* [20–22]. It is quite possible that, in *M. ramannianus*, an inordinate increase in the chitin synthetase activity and the irregular distribution of chitin in the cell wall due to the low level of ergosterol in germinating sporangiospores are responsible for the deformation of the mycelium.

From the results obtained, it may be concluded that, in order to assess the capacity of mucor fungi for yeastlike growth, it is necessary to take into consideration the following qualitative and quantitative characteristics of their sporangiospore lipids: (1) increased PE/PC ratio; (2) increased methylsterols/desmethylsterols ratio, as well as (3) low values of the PL/GL ratio and (4) low values of the ES/FS ratio. These results may be considered the criteria of sporangiospore capacity for giving rise to yeastlike growth during spore germination.

Hence, in mucor fungi, the capacity for yeastlike growth, which promotes the survival of dimorphic fungi under stress conditions, is associated with changes in the lipid content and composition, such as a decrease in the total lipid pool, in the percentage of ergosterol, phospholipids, and reserve lipids, as well as an increase in the percentage of glicolipids, precursors, and products of the ergosterol degradation. The mucor fungi whose sporangiospores contain high levels of reserve lipids (TAG and ES), as well as a high level of PC and ergosterol in the membrane and low level of methylated precursors of ergosterol, grow, for the most part, in the form of mycelium.

REFERENCES

1. Orlowski, M., *Mucor* Dimorphism, *Microbiol. Rev.*, 1991, vol. 5, no. 2, pp. 234–258.
2. Funtikova, N.S., Mysyakina, I.S., and Poglazova, M.N., Morphogenesis and Lipid Composition of *Mucor* Fungi Grown in the Presence of Chloroanilines in Submerged Culture, *Mikrobiologiya*, 1999, vol. 68, no. 4, pp. 467–472 [*Microbiology* (Engl. Transl.), vol. 68, no. 4, pp. 406–411].
3. Mysyakina, I.S. and Funtikova, N.S., Lipid Composition of the Yeastlike and Mycelial *Mucor hiemalis* Cells Grown in the Presence of 4-Chloroaniline, *Mikrobiologiya*, 2000, vol. 69, no. 6, pp. 790–795 [*Microbiology* (Engl. Transl.), vol. 69, no. 6, pp. 670–675].
4. Mysyakina, I.S. and Funtikova, N.S., Lipid Composition of the Arthrospores, Yeastlike Cells, and Mycelium of the Fungus *Mucor hiemalis, Mikrobiologiya*, 2001, vol. 70, no. 4, pp. 465–470 [*Microbiology* (Engl. Transl.), vol. 70, no. 4, pp. 403–408].
5. Mysyakina, I.S. and Funtikova, N.S., Changes in the Lipid Composition of *Mucor hiemalis* Sporangiospores Related to the Age of the Spore-Forming Culture, *Mikrobiologiya*, 2003, vol. 72, no. 4, pp. 516–520 [*Microbiology* (Engl. Transl.), vol. 72, no. 4, pp. 461–465].
6. Folch, G., Lees, M., and Sloane-Stanley, G.H., A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem.*, 1957, vol. 226, no. 1, pp. 497–509.
7. Kates, M., *Techniques of lipidology: isolation, analysis and identification of lipids*, Amsterdam: Elsevier, 1986, p. 464.
8. Mysyakina, I.S., Funtikova, N.S., and Medvedev, F.A., Sterol Composition of the Arthrospores and Mycelium of the Fungus *Mucor hiemalis, Mikrobiologiya*, 2002, vol. 71, no. 4, pp. 475–481 [*Microbiology* (Engl. Transl.), vol. 71, no. 4, pp. 404–409].
9. Weete, J.D., *Lipid Biochemistry of Fungi and Other Organisms*, New York, London: Plenum, 1980.
10. Funtikova, N.S. and Mysyakina, I.S., Sporangiospores of the Fungus *Mucor lusitanicus* 12M: Correlation between Lipid Composition, Viability, and Morphology of Growth upon Germination, *Mikrobiologiya*, 2003, vol. 72, no. 6, pp. 775–779 [*Microbiology* (Engl. Transl.), vol. 72, no. 6, pp. 686–689].
11. Morozova, E.V., Kozlov, V.P., Tereshina, V.M., Memorskaya, A.S., and Feofilova, E.P., Changes in Lipid Composition and Carbohydrate Composition of *Aspergillus niger* Conidia during Germination, *Prikl. Biokhim. Mikrobiol.*, 2002, vol. 28, no. 2, pp. 149–154 [*Appl. Biochem. Mikrobiol.* (Engl. Transl.), vol. 28, no. 2, pp. 129–133].
12. Mysyakina, I.S. and Funtikova, N.S., Sterols of the Fungus *Mucor hiemalis* Sporangiospores, *Mikrobiologiya*,

2003, vol. 72, no. 6, pp. 862–863 [*Microbiology* (Engl. Transl.), vol. 72, no. 6, pp. 762–763].

13. Vanden Bossche, H., Importance and Role of Sterols in Fungal Membranes, *Biochemistry of Cell Walls and Membranes in Fungi*, Kuhn, P.J., Trinci, A.P.J., Jung, M.J., Goosey, M.W., and Copping, L.G., Eds., Berlin: Springer, 1990, pp. 135–157.
14. Vanden Bossche, H., Marichal, P., le Jeune, L., Coene, M.-C., Gorrens, J., and Cools, W., Effects of Itraconazole on Cytochrome P-450-Dependent Sterol 14α-Demethylation and Reduction of 3-Ketosteroids in *Cryptococcus neoformans, Antimicrob. Agents Chemother.*, 1993, vol. 37, pp. 2101–2105.
15. Mercer, E.I., The Biosynthesis of Ergosterol, *Pestic. Sci.*, 1984, vol. 15, pp. 133–155.
16. Kelly, L., Lamb, D.C., Taylor, M., Corran, A.J., Baldwin, B.C., and Powderly, W.G., Resistance to Amphotericin B Associated with Defective Sterol $\Delta^{8\rightarrow7}$ Isomerase in a *Cryptococcus neoformans* Strain from An AIDS Patient, *FEMS Microbiol. Letts.*, 1994, vol. 122, pp. 39–42.
17. Pesti, M., Campbell, J.M., and Peberdy, J.F., Alteration of Ergosterol Content and Chitin Synthase Activity in *Candida albicans, Curr. Microbiol.,* 1981, vol. 5, pp. 187–190.
18. Chiew, Y.Y., Sullivan, P.A., and Shepherd, M.G., The Effect of Ergosterol and Alcohols on Germ-Tube Formation and Chitin Synthase in *Candida albicans, Can. J. Biochem.*, 1982, vol. 60, pp. 15–20.
19. Yarden, O. and Yanofski, C., Chitin Synthase I Plays a Major Role in Cell Wall Biogenesis in *Neurospora crassa, Genes Dev.*, 1991, vol. 5, pp. 2420–2430.
20. Kerkenaar, A. and Barug, D., Fluorescence Microscope Studies of *Ustilago maydis* and *Penicillium italicum* After Treatment with Imazalil Or Fenpropimorph, *Pestic. Sci.*, 1984, vol. 15, pp. 199–205.
21. Marishal, P., Gorrens, J., and Vanden Bossche, H., The Action of Itraconazole and Ketokonazole on Growth and Sterol Synthesis in *Aspergillus fumigatus* and *Aspergillus niger, Sabouraudia: J. Med. Vet. Mycol.*, 1985, vol. 23, pp. 13–21.
22. Hector, R.F. and Braun, P.C., The Effect of bifonazole on chitin synthesis in *Candida albicans, Recent trends in the discovery, development and evaluation of antifungal agents*, Fromtling, R.A. and Prous, J.R., Eds., Barcelona: Science Publishers, 1987, pp. 369–382.