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Resistance to Cellobiose Lipids and Specific Features of Lipid Composition in Yeast

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Abstract—The significance of the fatty acid composition and ergosterol content in cells for resistance to cellobiose lipids has been investigated in the cells of mutant *Saccharomyces cerevisiae* strains that are unable to produce ergosterol or sphingomyelin and in the cells of microorganisms that produce cellobiose lipids. *S. cerevisiae* mutants were shown to be less sensitive to cellobiose lipids from *Cryptococcus humicola* than the wild-type strain, and the strains that produced cellobiose lipids were virtually insensitive to this compound as well. The sensitivity of *Pseudozyma fusiformata* yeast to its own cellobiose lipids was reduced under conditions that favored the production of these compounds. No correlation between the content of ergosterol and sensitivity to cellobiose lipids was observed in *S. cerevisiae* or in the strains that produced cellobiose lipids. The ratio between the levels of saturated and unsaturated fatty acids in the cells of the mutant strains was correlated to the sensitivity of the cells to cellobiose lipids.

Keywords: yeast, cellobiose lipids, fungicide, ergosterol, sphingomyelin, fatty acids

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The diversity of secondary metabolites is characteristic of the kingdom of fungi. These compounds are not essential for metabolism; however, they provide the organisms that produce them with advantages for survival under unfavorable conditions and in the competition for ecological niches. Many antibiotic compounds are secondary metabolites of fungi. Certain yeast species secrete cellobiose lipids that exhibit fungicidal activity towards multiple species of yeast and fungi from different taxa, including the pathogens of human, animals, and plants [1–3]. Cellobiose lipids are detergents, and the biological activity of these substances is due to membrane disruption [4–6].

The sensitivity of different fungi and yeast species to these compounds varies considerably. The growth of ascomycetes and mycelial fungi is suppressed at much higher concentrations of these compounds than the growth of basidiomycetes [7]. For instance, almost all cells of the cryptococcosis pathogen *Filobasidiella neoformans* die after 30 minutes of incubation with 0.02 mg/mL cellobiose lipids, whereas the concentrations required to achieve the same effect in case of pathogenic *Candida* range from 0.16 to 0.2 mg/mL. Cellobiose lipids have considerable potential for the development of a new generation of fungicidal drugs, and therefore the research on factors that underlie the varying sensitivity of target cells to these compounds is of considerable interest.

The sensitivity of target cells to certain membrane-disrupting antibiotics is known to depend on the ratio of the relative abundances of saturated and unsaturated fatty acid residues in the phospholipids and on the content of sterols, ergosterol in particular, in the membranes [8, 9]. For example, ergosterol is the primary target of the antibiotic amphotericin B, and therefore the antibiotic sensitivity of cells of a certain species is directly related to the content of this component [10, 11]. There are some reports on the correlation of ergosterol content in the membranes to the sensitivity of the cells to nystatin and its derivatives [12–14]. However, the presence of ergosterol was supposed to have no effect on the sensitivity of fungi to the cellobiose lipid from *Pseudozyma flocculosa*, since oomycetes that do not synthesize ergosterol were still sensitive to this compound [15]. Therefore, the question of the role of ergosterol in the sensitivity of fungi to cellobiose lipids remains open. The resistance of cells to amphotericin B reportedly depends on the ratio of the relative abundances of saturated and unsaturated fatty acid residues in the membrane phospholipids [16].

The goal of the present work was to investigate the resistance of *S. cerevisiae* mutants with an unconventional lipid composition of the membrane, as well as the resistence of the organisms that produce cellobiose lipids, to two cellobiose lipids of different structure.

METHODS

Yeast strains and cultivation conditions. The *S. cerevisiae* strains used in the present work were the following: BY 4741 ("wild-type"; a kind gift from Dr. P. Kane, Upstate Medical University, United States) [17], lcb 1-100 [18], and erg 6 [19]. Yeast *Cryptococcus humicola* 9-6 [1] and *Pseudozyma fusiformata* [20] were used in the study as well.

S. cerevisiae were cultivated for 1 day at 30°C on a shaker in YPD medium that contained 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. *C. humicola* and *P. fusiformata* yeast were cultivated on a nutrient-depleted YPD-P medium that contained 10 g/L glucose, 5.0 g/L peptone produced by enzymatic hydrolysis (Sigma, United States), and 4.0 g/L yeast extract (Fluka, Germany). The nutrient medium used to stimulate cellobiose lipid production in *C. humicola* and *P. fusiformata* had the following composition: 10 g/L glucose, 1 g/L $(NH_4)_2SO_4$, 0.5 g/L yeast extract, and 0.05 g/L $MgSO_4 \cdot 7\ H_2O$. The microorganisms were cultivated on a shaker at 24°C for 2 days.

MIC assessment in microplates. The antibiotic activity of cellobiose lipids towards the studied yeast strains was assessed with the assay for minimal inhibitory concentration (**MIC**). The assay was performed in ELISA plates [21]. The cellobiose lipids used for MIC assessment were isolated from the culture liquid of *P. fusiformata* and *C. humicola* as described in [2, 3, 22]. The yeasts were maintained in the same media as the test cultures except for the addition of 0.04 M citrate-phosphate buffer, pH 4.0, to the media. The cultivation media, a cell suspension, and a methanol solution of cellobiose lipids were added to the wells of the ELISA plate such that the initial optical density of the sample ranged from 0.09 to 0.12 and the final lipid concentration was in the range of 0.01–0.3 mg/mL. The optical density of the suspensions was measured after 2–3 days of cultivation at 28°C in a thermostated shaker. A plate photometer (Sapfir, Russia) was used for the measurements. Methanol did not suppress yeast growth at the used concentrations. MIC was calculated as the concentration of glycolipids that induced a ~50% decrease of the optical density of the cultures.

Assessment of the composition of fatty acids. The biomass was lyophilized and subjected to acid methanolysis to assess the fatty acid composition [23]. The lyophilized biomass (500 ± 50 mg) was transferred into a flask and supplemented with 100 μL of the internal standard (heptadecanoic acid solution in chloroform), 10 mL of 10% hydrochloric acid solution in methanol, and 1 mL of anhydrous chloroform. The mixture obtained was refluxed at 80°C for 3 h and then extracted with a mixture of 7 mL hexane and 3 mL water and with two 7-mL portions of hexane. The top layer of the biphasic extract, which contained a mixture of methyl esters of fatty acids dissolved in hexane, was dehydrated by passing through calcined sodium sulfate and dried on a rotary evaporator under vacuum at 60°C. The obtained fatty acid methyl esters were analyzed by gas-liquid chromatography and identified with standard mixtures of methyl esters from Serva (Germany). The 200 × 0.3 cm column used for the analysis contained the Reoplex-400 sorbent (15%) on a Chromaton N-AW support (0.16–0.20 mm). The analysis was performed on a Chrom-5 device (Chrom, Czech Republic) in isothermal mode (170°C). Argon was used as the carrier gas, and anhydrous hexane was used as the solvent.

Assessment of sterol content. The method used for sterol extraction was a modification of the method described in [24–26]. Yeast cells (1.5–3.0 g wet biomass) were separated from the culture liquid by centrifugation at 5000 *g* and rinsed twice with distilled water. The cells were incubated with 15 mL of 20% KOH solution in methanol for 1 h at 85°C. The total sterols were extracted from the mixture by 5 to 10 mL of petroleum ester (the extractant volume was adjusted to the final volume of the sample). Most of the upper phase (70–80% of the volume) that contained sterols was collected, dried in a flow of nitrogen, and dissolved in methanol (2–3 mL).

The sterols were quantitated as described in [24, 27]. The optical density of the solutions at 230 nm (the absorption maximum for 24(28) dehydroxyergosterol (24(28) DHE), the metabolic precursor of ergosterol) and 280 nm (the absorption maximum for the complex of 24(28) DHE and ergosterol) was determined with a Beckman Coulter BH 730 spectrophotometer (Beckman, the United States). The relative content of 24(28) DHE (%) was calculated with the formula:

$$\%\ 24(28)\ \text{DHE} = [(\text{Abs}_{230}/518) \times F]/\text{pw},$$

where F is the dilution factor that takes the organic phase volume and the methanol solution volume into account and pw is the weight of the wet biomass. The relative content of ergosterol (%) was calculated with the formula:

$$\%\ \text{ergosterol} = [(\text{Abs}_{280}/290) \times F]/\text{pw} - [(\text{Abs}_{230}/518) \times F]/\text{pw},$$

where F is the dilution factor that accounts for the organic phase volume and the methanol solution volume and pw is the weight of the wet biomass.

The experiments were performed in three biological and three analytical replicates. The mean values and standard deviations are shown in the tables.

RESULTS AND DISCUSSION

Two cellobiose lipids of different composition were obtained; the structures of these lipids are shown in Fig. 1. Cellobiose lipids of *C. humicola* were represented by a mixture of 2,3,4-O-triacetyl-β-D-glucopyranosyl-(1→4)-6-O-acetyl-β-D-glucopyranosyl-(1→16)-2,16-dihydroxyhexadecanoic acid and 2,3,4-O-triacetyl-β-D-glucopyranosyl-(1→4)-6-O-acetyl-

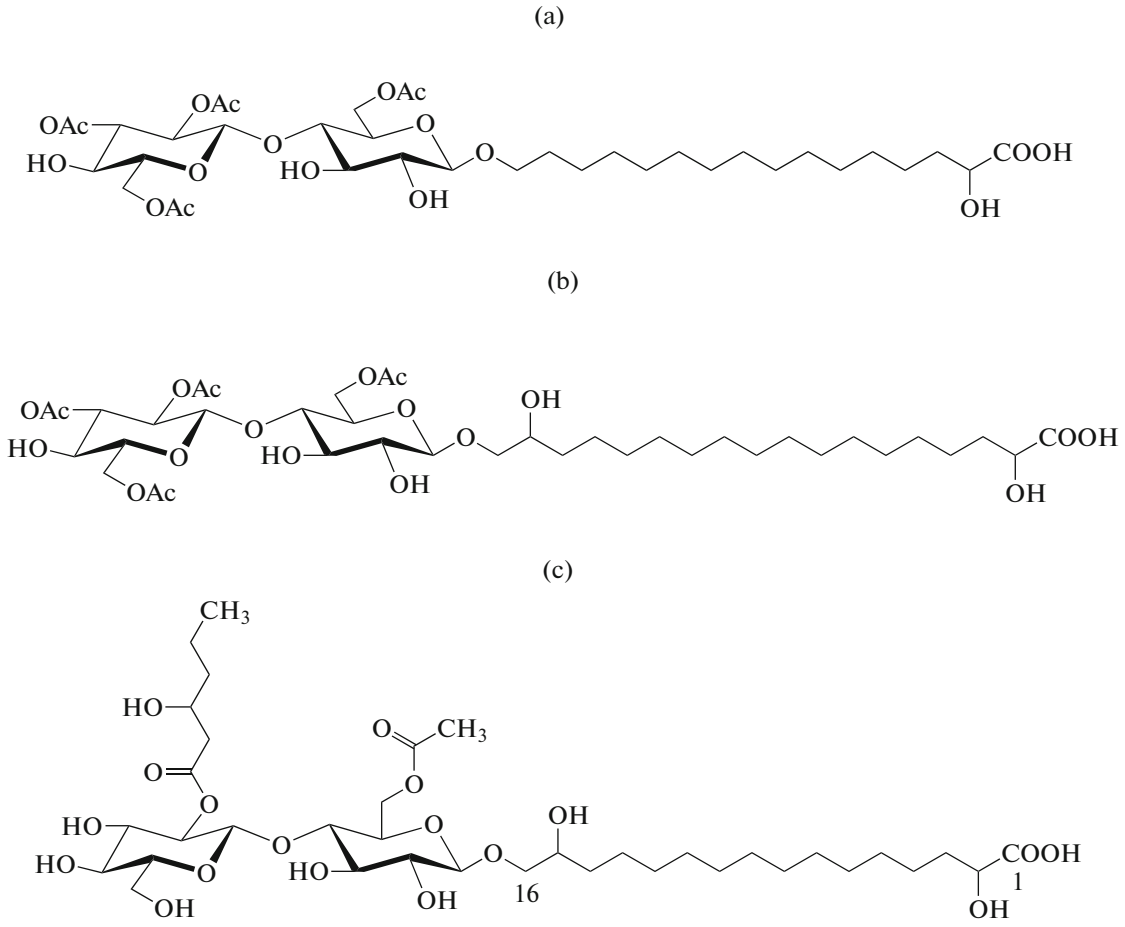

Fig. 1. Structure of the major cellobiose lipids produced by *C. humicola* (a, b) and *P. fusiformata* (c).

β-D-glucopyranosyl-(1→16)-2,15,16-hydroxyoctadecanoic acid [22] (Figs. 1a and 1b). The cellobiose lipid obtained from the culture liquid of *P. fusiformata* was identified as 2-O-3-hydroxyhexanoyl-β-D-glucopyranosyl-(1→4)-6-O-acetyl-β-D-glucopyranosyl-(1→16)-2,15,16-trihydroxyhexadecanoic acid [3] (Fig. 1c).

The previous study demonstrated similar fungicidal activity of two cellobiose lipids with different structures towards different yeast species and the ability of these lipids to induce complete elimination of fungal cells [7]; however, the fungicidal activity of the cellobiose lipid from *P. fusiformata* towards mycelial fungi was higher than that of the other lipid. The present study showed that *S. cerevisiae* with impairments in the pathways of sphingomyelin or ergosterol biosynthesis (lcb 1-100 and erg 6, respectively) were less sensitive to cellobiose lipids from *C. humicola* than the wild-type strain BY 4741 (Table 1). However, the effects of the cellobiose lipid from *P. fusiformata* on these mutant strains were similar to the effect on the wild-type strain (Table 1). The cellobiose lipid of *C. humicola* did not suppress the growth of the source strain or *P. fusiformata*, even at a concentration of 0.3 mg/mL. The use of higher concentrations was limited by the solubility of the cellobiose lipid in the cultivation medium, and conclusive results could therefore not be obtained. The cellobiose lipid produced by *P. fusiformata* suppressed the growth of *C. humicola* yeast at the concentration of 0.08 mg/mL. The effect was observed with cultivation on glucose-peptone medium, as well as with cultivation on a nitrogen-depleted medium that stimulated the synthesis of cellobiose lipids. Notably, this lipid could suppress the growth of ascomycete yeast when used at the same concentration of 0.08 mg/mL. The growth of yeast *P. fusiformata* was suppressed by the cellobiose lipid produced by this species. However, the suppression observed under conditions that favored cellobiose lipid synthesis was less pronounced than the suppression of yeast growth on glucose-peptone medium (Table 1). The higher efficiency of the cellobiose lipid from *P. fusiformata* is probably related to the presence of an additional fatty acid residue in the cellobiose moiety of this glycolipid.

The ergosterol content in cells of the *erg 6* strain is reportedly lower than that in cells of the wild-type *S. cerevisiae* strain [19], whereas the ergosterol content

Table 1. Sensitivity of different *S. cerevisiae* strains and the microorganisms that produce glycolipids to cellobiose lipids

Species, strain	Cultivation medium	MIC, mg/mL	
		C. humicola cellobiose lipid	*P. fusiformata* cellobiose lipid
S. cerevisiae BY 4741 (wild type)	YPD	0.04	0.04
S. cerevisiae lcb 1-100	YPD	0.3	0.04
S. cerevisiae erg 6	YPD	0.3	0.04
C. humicola 9-6	YPD-P	Insensitive at 0.3 mg/mL	0.08
C. humicola 9-6	Medium with $(NH_4)_2SO_4$	"	0.08
P. fusiformata VKM Y-2821	YPD-P	"	0.08
P. fusiformata VKM Y-2821	Medium with $(NH_4)_2SO_4$	"	0.15

Table 2. Ergosterol content in the cells of different *S. cerevisiae* strains and in the cells of microorganisms that produce cellobiose lipids

Species, strain	Cultivation medium	Ergosterol, %
S. cerevisiae BY 4741 (wild type)	YPD	0.043 ± 0.0012
S. cerevisiae lcb 1-100	YPD	0.061 ± 0.0006
S. cerevisiae erg 6	YPD	0.014 ± 0.0006
C. humicola 9-6	YPD-P	0.023 ± 0.0005
P. fusiformata VKM Y-2821	YPD-P	0.022 ± 0.0017
P. fusiformata VKM Y-2821	Medium with $(NH_4)_2SO_4$	0.029 ± 0.0006

in cells of the strain *lcb1-100,* which is incapable of sphingomyelin synthesis, was higher than that in cells of the wild-type strain (Table 2). The ergosterol content in cells of the microorganisms that produced cellobiose lipids was lower than that in the wild-type *S. cerevisiae* strain BY4741 but higher than that in the *erg 6* strain; the difference did not depend on the type of cultivation medium used.

The content of C18 fatty acid with two double bonds in the molecule was higher in the wild-type *S. cerevisiae* cells (strain BY 4741) than in cells of the strains *erg 6* and *lcb1-100* (Table 3). Cells of the wild-type strain contained a monounsaturated C17 fatty acid that was virtually absent from the cells of other strains investigated, whereas the content of the saturated C16 fatty acid in the wild-type cells was much lower than that in the cells of mutant strains.

The fatty acid composition in the cells of microorganisms that produced cellobiose lipids was characterized by a high abundance of saturated fatty acids and differed considerably from the composition of fatty acids in the cells of *S. cerevisiae*. The cells of both species that produced cellobiose lipids lacked the monounsaturated C16 fatty acid under all of the used cultivation conditions. *P. fusiformata* cells grown under conditions that favored the production of cellobiose lipids did not contain detectable amounts of the saturated C18 fatty acid, which was present in cells grown on glucose-peptone medium (Table 4).

Thus, the absence of a correlation between the sensitivity of the cells of mutant strains to cellobiose lipids and the presence of ergosterol in the cells was evident from the results. The reduced sensitivity of the mutant strains to cellobiose lipids is apparently related to the specific composition of fatty acids in the cells, especially to the predominance of saturated fatty acid residues, which leads to a decrease in membrane fluidity [28, 29]. These results strengthened the earlier hypothesis of the absence of specific targeting of ergosterol in the cytoplasmic membrane by cellobiose lipids [15]. The changes in fatty acid composition in the mutant strains may be due to the pleiotropic effects of mutations in genes involved in the synthesis of ergosterol or sphingomyelin.

The high content of saturated and monounsaturated C18 fatty acids and the saturated C16 fatty acid in *C. humicola* cells apparently determined the insensitivity of glycolipid-producing organisms to the cellobiose lipid products of their own metabolism. The increased resistance of *P. fusiformata* to its own cellobiose lipids in the case of cultivation under conditions favoring glycolipid production (nitrogen deficit) is apparently not related to changes in the lipid composition of the yeast cells. Indeed, the ergosterol content did not change under these conditions, although the content of monounsaturated C18 fatty acids decreased and the saturated C18 fatty acid disappeared, leading to an increase in membrane fluidity. The decrease of the sensitivity of *P. fusiformata* to the cellobiose lipid

Table 3. Content of fatty acids in the cells of different *S. cerevisiae* strains

Fatty acid, % of total content	Species, strain		
	S. cerevisiae BY 4741 (wild type)	*S. cerevisiae* lcb 1-100	*S. cerevisiae* erg 6
C14	1.25 ± 0.06	0.3	0
C16	7.45 ± 0.06	15.4	18.4 ± 0.58
C16:1	29.8 ± 0.46	33.9 ± 0.06	18.5 ± 0.17
C17	0.5 ± 0.17	0.5 ± 0.35	1.6 ± 0.23
C17:1	3.1 ± 0.06	0.85 ± 0.52	0
C18	2.8 ± 0.12	4.1 ± 0.17	19.9 ± 2.1
C18:1	26.7 ± 0.17	35.3 ± 0.12	36.6 ± 0.8
C18:2	24.5 ± 0.4	9.65 ± 0.52	2.9 ± 0.29
C18:3	3.9 ± 0.06	0	Tr.
C20	0	0	2.1 ± 0.8

Table 4. Content of fatty acids in the cells of microorganisms that produced cellobiose lipids

Fatty acid, % of total content	Species and strains		
	C. humicola 9-6 (YPD-P medium)	*P. fusiformata* BKM Y-2821 (YPD-P medium)	*P. fusiformata* BKM Y-2821 (medium with $(NH_4)_2SO_4$)
C14	1.5 ± 0.06	0.65 ± 0.06	1.9 ± 0.06
C16	30.0 ± 0.12	21.1 ± 0.52	24.8 ± 1.04
C16:1	0	0	0
C17	1.3 ± 0.12	2.15 ± 0.06	0.15 ± 0.06
C17:1	0	0	0
C18	10.1 ± 0.17	10.2 ± 0.12	0
C18:1	47.0 ± 0.06	46.0 ± 0.87	56.7 ± 0.8
C18:2	9.4	19.4	15.6 ± 017
C18:3	0.7	0	0
C20	0	0.5 ± 0.29	0.85 ± 0.06

under cultivation conditions that stimulate the synthesis of this lipid may be related to the induction of enzymes that catabolize cellobiose lipids [30].

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