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EXPERIMENTAL ARTICLES

The Yeast *Pseudozyma fusiformata* VKM Y-2821 Producing an Antifungal Glycolipid

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Abstract—The yeast *Pseudozyma fusiformata* (*Ustilaginales*) produces an extracellular low-molecular-weight protease-resistant thermostable fungicide, which is active against more than 80% of the 280 yeast and yeast-like species tested under acidic conditions. The fungicide, extracted with methanol and purified by column and thin-layer chromatography, was found to consist of glucose and saturated fatty acids.

Key words: glycolipid, fungicide, *Ustilaginales*, antagonism.

Many microorganisms are known to produce antibiotics. Some yeasts, called killer yeasts, secrete into the medium mycocins, which are most commonly glycosylated proteins with a molecular mass of about 20 kDa possessing either fungicidal or fungistatic activity. Basically, mycocins act against the microorganisms that are phylogenetically related to the producing strain. In other words, the action of mycocins is taxonomically specific [1]. However, some killer strains of *Cryptococcus humicola* produce unidentified chemical compounds that are active against a broad range of phylogenetically distant ascomycetous and basidiomycetous yeasts [2]. Unlike common mycocins, the antifungal agents of *Cr. humicola* have a low molecular mass (about 1 kDa) and are thermostable and protease-resistant. It is evident that antagonistic relations between yeasts can be caused not only by mycocins but also by other antifungal agents.

The yeast *Pseudozyma fusiformata* (Buhagiar) Boekhout of the order *Ustilaginales* has a high antibiotic activity. The aim of the present work was to characterize this activity and the substance responsible for it.

MATERIALS AND METHODS

The test cultures used in this work were obtained from the Russian Collection of Microorganisms (VKM), Japan Collection of Microorganisms (JCM), and the collection of the Meiji College of Pharmacy (Japan). The antifungal activity of *Ps. fusiformata* VKM Y-2821 was assayed at 20°C by the culture-to-culture method using a glucose–peptone agar medium prepared with a sodium succinate buffer (pH 4.5) [2]. In the case of test cultures from the genus *Malassezia*, the medium was supplemented with olive oil (10 ml/l).

To obtain antifungal agent, strain VKM Y-2821 was grown in a liquid glucose–peptone medium (pH 4.5) for one month at 24°C. Cells were removed by centrifugation at 3000 *g* for 20 min, and the supernatant was passed through GF/A filters (Whatman, United Kingdom). The culture liquid filtrate with antibiotic activity was assayed for its thermostability and resistance to the action of proteases by the agar well method. The antifungal agent was extracted from the lyophilized filtrate with methanol. The extract was dried at 30–40°C using a vacuum rotary evaporator and the residue was suspended in distilled water with pH adjusted to 8.0 by the addition of NaOH. The precipitate obtained from this suspension by filtration was dissolved in methanol and purified twice by gel filtration on LH-Sephadex (Pharmacia, Sweden) in methanol. The eluate was collected in 10-ml fractions. To assay the antifungal activity of these fractions, their aliquots were placed, in impregnated 5-mm-diameter GF/A filters, onto a *Cryptococcus terreus* VKM Y-2253 lawn grown on glucose–peptone agar (pH 4.5). The active fractions were pooled, concentrated using the vacuum rotary evaporator, and purified by thin-layer chromatography on Silufol plates (Kavalier, Czech Republic) in a chloroform–methanol–water (10 : 10 : 0.4) system. Separated substances were visualized by spraying the developed plates with solutions of H_2SO_4 and ninhydrin or incubating them in iodine vapors [3].

Pertinent spots were scraped off the plates and eluted with methanol. The methanol extracts were hydrolyzed in 4 N HCl and 3 M trifluoroacetic acid at 100°C for 10 h. The hydrolysates were analyzed for the content of amino acids and carbohydrates using an AAA-881 amino acid analyzer (Microtechna, Czech Republic) and an LC-2000 carbohydrate analyzer (Biotronic, Germany). The fatty acid composition of

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Yeast and yeast-like microorganisms susceptible to the glycolipid of *Pseudozyma fusiformata* VKM Y-2821

Agaricostilbum (1, 1)	*Mrakia* (2, 3)
Arthroascus (1, 1)	*Myxozyma* (4, 4)
Arxula (1, 1)	*Nadsonia* (1, 1)
Bensingtonia (6, 6)	*Pichia* (1, 1)
Bullera (11, 30)*	*Platygloea* (1, 1)
Bulleromyces (1, 1)	*Protomyces* (1, 1)*
Candida (2, 2)*	*Puccinia* (1, 1)*
Cryptococcus (33, 33)*	*Rhodosporidium* (1, 1)*
Cystofilobasidium (4, 4)	*Rhodotorula* (34, 43)*
Debaryomyces (7, 7)*	*Saccharomyces* (1, 1)*
Dipodascus (1, 1)	*Septobasidium* (1, 1)
Entyloma (1, 1)	*Sorosporium* (1, 1)
Erythrobasidium (1, 1)	*Sphacelotheca* (1, 1)
Exobasidium (6, 8)	*Sporidiobolus* (3, 3)*
Farysia (1, 1)	*Sporisorium* (2, 3)
Fellomyces (3, 3)	*Sporobolomyces* (22, 25)*
Filobasidiella (1, 44)*	*Stephanoascus* (1,1)
Filobasidium (2, 4)*	*Sterigmatomyces* (2, 2)
Guilliermondella (1, 1)	*Sympodiomycopsis* (1, 1)
Gymnosporangium (1, 1)	*Taphrina* (8, 8)*
Holtermannia (1, 1)	*Tausonia* (1, 1)
Issatchenkia (1,1)	*Tilletiaria* (1, 1)
Itersonilia (1, 2)	*Tilletiopsis* (7, 9)
Kockovaella (1, 1)	*Torulaspora* (1, 1)
Kurtzmanomyces (1, 1)	*Trichosporon* (11, 12)
Leucosporidim (2, 2)	*Trimorphomyces* (1, 1)
Lipomyces (3, 3)*	*Udeniomyces* (3, 12)
Lodderomyces (1,1)	*Ustilago* (10, 12)*
Malassezia (2, 2)	*Wickerhamia* (1, 1)
Mastigobasidium (1, 1)	*Xanthophyllomyces* (1, 4)
Mastigomyces (1, 1)	*Zygozyma* (1, 1)
Microbotryum (4, 4)*	

* Parenthesized are the numbers of tested species and strains (see text). The asterisks mark the taxa than are heterogeneous with respect to the action of the glycolipid.

the antifungal agent was determined by gas–liquid chromatography as described elsewhere [4, 5].

RESULTS

The antibiotic activity of *Ps. fusiformata* VKM Y-2821 was first revealed against yeast-like fungi of the genus *Tilletiopsis.* This activity was observed only at pH values between 3.5 and 6.0, being highest at pH 4.5–5.0 in sodium succinate buffer.

The fungicidal agent of strain VKM Y-2821 (see Fig. 1) exhibited a broad action spectrum: it was active against 83% of the tested 280 fungal species (411 strains) belonging to 80 genera (see table). At the same time, 28 species (42 strains) of the genera *Ambrosiozyma, Atractogloea, Calocera, Citeromyces, Clavispora, Dacrymyces, Endophyllum, Guepiniopsis, Hanseniaspora, Neovossia, Pseudozyma, Saturnispora, Schizosaccharomyces, Tilletia, Tsuchiyaea, Williopsis,* and *Zygosaccharomyces* were found to be resistant to this agent. Some taxa turned out to be heterogeneous with respect to the action of the fungicide. For instance, the species *C. glabrata, Cr. flavus, Cr. hempflingii, Cr. huempii, F. capsuligenum, Lip. tetrasporus, Mic. scorzonerae, Mic. violaceum, Pr. macrosporus, Puc. bupleuri, R. lusitaniae, R. sphaerocarpum, Rh. colostri, Rh. bogoriensis, Rh. nothofagi, S. salmonicolor, Sp. inositophilus, Sp. singularis, Taph. purpurescens, Taph. robinsoniana, Taph. sadebeckii, Taph. tasquinetii, Tr. gracile,* and *U. maydis* were resistant to the fungicide of strain VKM Y-2821, whereas other species of the respective genera were susceptible to it (see table).

Some species were also heterogeneous. For instance, only seven of eleven *B. alba* strains, one of four *B. pseudoalba* strains, one of three *Cr. laurentii* strains, 44 of 47 *F. neoformans* strains, and one of two *Sacch. cerevisiae* strains were found to be susceptible to the *Ps. fusiformata* fungicide. The heterogeneity of two species coincided with their division into varieties. Namely, *Deb. hansenii* var. *hansenii* and *Deb. polymorphus* var. *polymorphus* were sensitive, whereas *Deb. hansenii* var. *fabryi* and *Deb. polymorphus* var. *africanus* were insensitive to the fungicide.

As judged from the size of the growth inhibition zone, the fungicide of *Ps. fusiformata* retains its activity after 90 min of incubation at 100°C. It is also resistant to the action of pronase E (Sigma, United States) and lysoamidase, a broad-spectrum bacteriolytic complex possessing protease activity [6]. Experiments with ultramembranes with different pore sizes (Spectrum, United States) showed that the fungicide passed through membranes with a nominal molecular weight cut-off of 3500 Da. The gel filtration of the methanol extract of the lyophilized culture liquid filtrate of strain VKM Y-2821 on LH-Sephadex in methanol showed the presence of fungicidal activity only in the first peak (chromatographic fractions 7 and 8), whose elution volume was close to that of actinomycin D (molecular mass 1255 Da) (Fig. 2). Therefore, the antifungal agent of *Ps. fusiformata* has a molecular mass of about 1 kDa.

The thin-layer chromatography of the active fraction obtained by gel filtration on LH-Sephadex showed the presence of fungicidal activity in the substance with an R_f value of 0.8 (Fig. 3). This substance stained with H_2SO_4 or iodine vapors, but not with ninhydrin. Analyses showed that it does not contain amino acids but contains glucose and traces of cellobiose, as well as the saturated fatty acids C12:0, C14:0, and C16:0 in relative amounts of, respectively, 7.3, 36.4, and 56.2% of the total fatty acids.

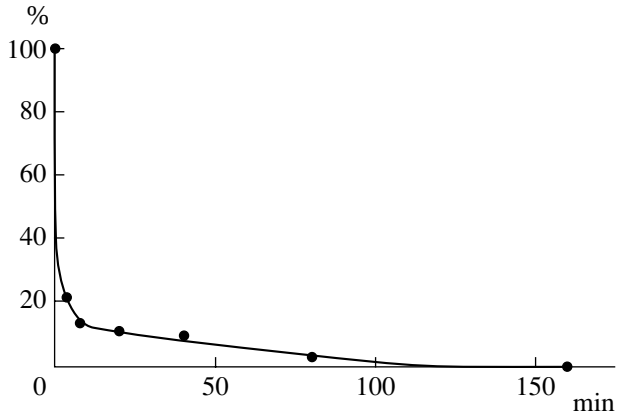

Fig. 1. The death kinetics of *Ustilago perennans* VKM F-2972 cells (the initial concentration of 10^4 cells/ml is taken to be 100%) incubated at 20°C with the lyophilized culture liquid of *Pseudozyma fusiformata* VKM Y-2821 (6 mg/ml) in a medium with pH 4.5. The number of viable cells was evaluated by plating the cell suspension dilutions onto wort agar plates taken in triplicate.

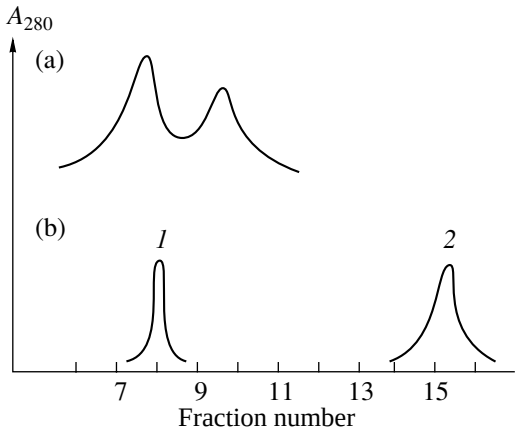

Fig. 2. Elution profiles of (a) the methanol extract of the lyophilized culture liquid of *Pseudozyma fusiformata* VKM Y-2821 and (b) marker antibiotics subjected to gel filtration on LH-Sephadex in methanol (flow rate, 10 ml/h; fraction volume, 3 ml): (*1*) actinomycin D (M_r = 1255 Da) and (*2*) oxytetracycline (M_r = 496 Da).

DISCUSSION

The physicochemical data obtained suggest that the extracellular fungicidal agent of *Ps. fusiformata* VKM Y-2821 is a glycolipid whose carbohydrate moiety presumably represents cellobiose, which is hydrolyzed to glucose during analysis. The lipid moiety of the glycolipid consists mainly of myristic and palmitic acids. The formation of extracellular glycolipids by yeast and yeast-like fungi has long been known [7–9], but the glycolipid of strain VKM Y-2821 differs from the known yeast glycolipids in the composition of either carbohydrate or lipid moiety. In the carbohydrate moiety, this glycolipid is most close to the cellobiosolipid of *Ustilago maydis.*

Thus, in its chemical nature, the fungicide of *Ps. fusiformata* is not a mycocin. This fact emphasizes once again that mycocinogeny should be distinguished from other growth-inhibiting phenomena [1].

Yeast glycolipids are often considered to be surfactants responsible for the uptake of hydrophobic substrates [8, 9]. However, as early as in 1980, Ito *et al.* showed that the sophorolipid of *Candida bombicola* can either inhibit or stimulate the growth of some yeasts on hydrocarbons at pH 5.6 [10]. Taking into account that the glycolipid of *Ps. fusiformata* is most active at pH 4.5, the possibility that pH 5.6 is not optimum for the antibiotic activity of the sophorolipid cannot be excluded.

Unlike yeast glycolipids, the cellobiosolipid of *U. maydis* (ustilagic acid) is a broad-range antifungal agent possessing even antibacterial activity [11], although the latter activity may be due to the acidic properties of the cellobiosolipid. Similar to the glycolipid of *Ps. fusiformata,* ustilagic acid is thermostable. As for the pH dependence of its antibiotic activ-

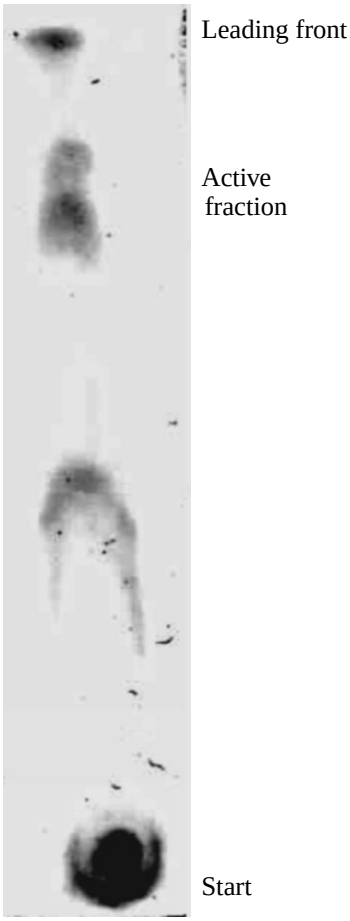

Fig. 3. Thin-layer chromatography of the fungicidal agent purified by column chromatography on LH-Sephadex in methanol. The developed plate was sprayed with 15% H_2SO_4 in 95° ethanol.

ity, it is unknown. Interestingly, only one of the 11 tested species of the genus *Ustilago* (see table), *U. maydis*, is resistant to the glycolipid of *Ps. fusiformata*. This fact is easily understandable if we take into account the chemical similarity of the fungicidal glycolipids of these two species.

The low toxicity of fungal glycolipids to vertebrates [11, 12] and the broad-range activity of the glycolipids of *Ps. fusiformata* and *U. maydis* against pathogenic fungi allow one to consider them promising fungicides.

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REFERENCES

1. Golubev, W.I., Mycocins (Killer Toxins), *The Yeasts: A Taxonomic Study*, Kurtzman, C.P. and Fell, J.W., Eds., Amsterdam: Elsevier, 1998, pp. 55–62.
2. Golubev, W. and Shabalin, Y., Microcin Production by the Yeast *Cryptococcus humicola*, *FEMS Microbiol. Lett.*, 1994, vol. 119, nos. 1–2, pp. 105–110.
3. Kates, M., *Techniques of Lipidology: Isolation, Analysis and Identification of Lipids*, Amsterdam: Elsevier, 1972. Translated under the title *Tekhnika lipidologii*, Moscow: Mir, 1975.
4. Eroshin, V.K., Satroutdinov, A.D., Dedyukhina, E.G., and Chistyakova, T.J., Arachidonic Acid Production by *Mortierella alpina* with Growth-coupled Lipid Synthesis, *Process Biochem.*, 2000, vol. 35, no. 8, pp. 1171–1175.
5. Sultanovich, Yu.A., Nechaev, A.P., and Barsukova, I.A., Method for the Quantification of the Fatty Acid Composition of Microbial Lipids, USSR Inventor's Certificate no. 968072, *Byull. Izobret.*, 1982, no. 39, p. 136.
6. Stepnaya, O.A., Severin, A.I., and Kulaev, I.S., The Bacteriolytic Enzymes of Lysoamidase Isolated from Bacteria of the Family *Pseudomonadaceae*, *Biokhimiya*, 1986, vol. 51, no. 7, pp. 1117–1123.
7. Spencer, J.F.T., Spencer, D.M., and Tulloch, A.P., Extracellular Glycolipids of Yeasts, *Economic Microbiology: Secondary Products of Metabolism*, Rose, A.H., Ed., London: Academic, 1979, vol. 3, pp. 523–540.
8. Lang, S. and Wagner, F., Structure and Properties of Biosurfactants, *Biosurfactants and Biotechnology*, Kosaric, N., Cairns, W., and Gray, N.C.C., Eds., New York: Marcel Dekker, 1987, pp. 21–45.
9. Rosenberg, E. and Ron, E.Z., High- and Low-Molecular-Mass Microbial Surfactants, *Appl. Microbiol. Biotechnol.*, 1999, vol. 52, no. 2, pp. 154–162.
10. Ito, S., Kinta, M, and Inoue, S., Growth of Yeasts on *n*-Alkanes: Inhibition by a Lactonic Sophorolipid Produced by *Torulopsis bombicola*, *Agric. Biol. Chem.*, 1980, vol. 44, no. 9, pp. 2221–2223.
11. Haskins, R.H. and Thorn, J.A., Biochemistry of the *Ustilaginales*: VII. Antibiotic activity of ustilagic acid, *Can. J. Botany*, 1951, vol. 29, no. 6, pp. 585–592.
12. Ikeda, Y., Sunakawa, T., Tsuchiya, S., Kondo, M., and Okamoto, K., Toxicological Studies on Sophorolipid Derivatives, *J. Toxicol. Sci.*, 1986, vol. 11, no. 3, pp. 197–211.