ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 2, pp. 171–175. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © W.I. Golubev, T.V. Kulakovskaya, A.S. Shashkov, E.V. Kulakovskaya, N.V. Golubev, 2008, published in Mikrobiologiya, 2008, Vol. 77, No. 2, pp. 201–206.

EXPERIMENTAL ARTICLES

Antifungal Cellobiose Lipid Secreted by the Epiphytic Yeast *Pseudozyma graminicola*

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Received January 18, 2007; in final form, June 18, 2007

Abstract—The yeast *Pseudozyma graminicola* isolated from plants inhibited growth of almost all ascomycetes and basidiomycetes tested (over 270 species of ca. 100 genera) including pathogenic species. This yeast secreted a fungicidal agent, which was identified as a glycolipid composed of cellobiose residue with two O-substituents (acetyl and 3-hydroxycaproic acid) and 2,15,16-trihydroxypalmitic acid. The release of ATP from the glycolipid-treated cells indicated that this glycolipid impaired the permeability of the cytoplasmic membrane. Basidiomycetes were more sensitive to the cellobiose lipid than ascomycetes.

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

DOI: 10.1134/S0026261708020082

Saprophytic yeastlike fungi are abundant on plants and plant debris. In the phyllosphere, their amount may exceed 0.5 million cells/g; they are often a predominant component of the mycobiota [1]. Contrary to the generally accepted concept of epiphytic yeasts as neutral commensals, recent results are indicative of their ability to secret antifungal substances [2] and thus contribute to plant protection against pathogenic fungi [3].

Basidiomycetous yeastlike fungi belonging to the genus *Pseudozyma* Bandoni *emend*. Boekhout are widespread in the phylloplane; their antifungal activity can be due to the formation of both glycolipids [2] and mycocins [4]. We revealed the occurrence of representatives of this genus, along with the representatives of *Bullera* Derx., *Cryptococcus* Vuillemin, *Rhodotorula* Harrison, and *Sporobolomyces* Kluyver et van Niel, on gramma grasses (vetch, cockfoot, clover, goat's-rue, alfalfa, fescue, rye-grass, and timothy-grass). When the samples were plated on glucose–peptone agar (GPA), the number of grown propagules of mycelial and yeastlike fungi ranged from 5 to 84 and from 6 to 95 thousand/g, respectively. We identified three isolates of the genus *Pseudozyma* (L1-16, L1-41, and L1-71) as *P. fusiformata* (Buhagiar) Boekhout [5], whereas two other isolates (L1-20 and L1-46) were assigned to the novel species *P. graminicola* Golubev et al. [6]. The latter species was found to possess antifungal activity. The conditions of exhibiting antifungal activity, the chemical nature of the antifungal factor, and the spectrum of sensitive organisms are described in this work.

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MATERIALS AND METHODS

Strains. The strains L1-20 (VKM Y-2938) and L1-46 were isolated from cereal grasses (collected in June 2002 in the Mytishchi region, Moscow oblast) by plating the samples on glucose–peptone agar (GPA) supplemented with penicillin (1 million U/l) or streptomycin (1 g/l). The isolates were maintained on malt agar (MA) and stored at 5°C. Antifungal activity of isolates was determined by using the cultures obtained from the All-Russian Collection of Microorganisms (VKM, http://www.vkm.ru).

Sensitivity asaay. Three-day test cultures grown on MA at 20°C were used for the test. The sensitivity to the antifungal agent was determined by the culture-to-culture method on GPA with either citrate–phosphate or sodium succinate buffers (pH 4.0) incubated at room temperature [7]. The viability of yeasts after their incubation with the antifungal agent was assayed by plating cell suspensions on GPA and a colony count.

Elimination of antifungal activity. To determine the elimination of antifungal activity, 0.1 ml of cell suspensions (10^4 cells/ml) of strains L1-20 and L1-46 was plated on MA as a lawn and incubated at 35°C for two

weeks; the grown colonies were randomly chosen and tested for antifungal activity.

Preparation, isolation, and purification of the antifungal agent. Strains L1-20 and L1-46 were grown under static conditions for four weeks at 24°C on medium containing the following (g/l): glucose, 10.0; $(NH_4)_2SO_4$, 1.0; yeast extract, 0.5; $MgSO_4 \cdot 7H_2O$, 0,05; $Na_2HPO_4 \cdot 12H_2O$, 13.8; citric acid monohydrate, 6.45; pH 4.0. The cells were separated by centrifugation (5000 *g*, 30 min); the supernatant was passed through a CF/A filter (Whatman, Great Britain) and lyophilized. The lyophilized material obtained from 3 l of the culture medium was treated with 400–500 ml of methanol at 4°C for four to five days and then filtered through a glass filter; the methanol extract was concentrated to half of the initial volume on a rotary evaporator at 40–50°C. After removing the salt sediment by filtration, the extract was evaporated nearly to dryness. The residue was diluted with 250 ml of deionized water, and the solution was kept at 4°C overnight. The precipitate was separated by filtration through a glass filter, washed twice with deionized water at 4°C, and dissolved in 15–20 ml of methanol. At every stage, the antifungal activity of the preparations was tested as follows: aliquots (10–50 μl) were transferred to filter paper discs (5 mm in diameter), dried, and plated onto GPA (pH 4.0) plates with a lawn of *Cryptococcus terreus* VKM Y-2253 cells. The purity of the preparations was determined by thin-layer chromatography (TLC) on Kieselgel 60 F_{254} plates (Merck, Germany) developed in a chloroform–methanol–water (4 : 4 : 0.2) system. To visualize the antifungal agent, the plates were sprayed with a 5% solution of H_2SO_4 in 95% ethanol and dried at 100°C.

Methanolysis. The methanol solution of the preparation (20–100 mg) was dried in a flow of nitrogen, supplemented with acetyl chloride (6–8 drops) and 1–1.5 ml of methanol (4°C), and hydrolyzed at 90°C for 1 h. The hydrolysate was dried under vacuum and washed with 2–3 ml of hexane; the residue was dissolved in 1–2 ml of a mixture of chloroform and methanol (1 : 1). The obtained solution was analyzed by TLC as described above.

NMR spectroscopy and mass spectrometry. The preparations dried in a flow of nitrogen were diluted in pyridine-d_5 and their ^1H- and ^{13}C-NMR spectra were registered on a Bruker DRX-500 spectrometer (Germany) with tetramethylsilane (TMS) as an internal standard. Two-dimensional spectra (COSY, TOCSY, HSQC, and HMBC) were recorded by using the standard Bruker methods (the XWINNMR 1.2 software package). The mixing time in the TOCSY experiment was 0.1 s. The HMBC experiment was optimized for a constant $J_{C,H}$ of 8 Hz.

The mass spectra of the preparations diluted in methanol were registered on a LCO DECAXP Thermo Finnigan mass spectrometer (United States).

ATP determination was performed by a luminometric method [8].

RESULTS AND DISCUSSION

Antifungal activity of strains L1-20 and L1-46 was revealed after their screening on GPA (pH 4.5) with *C. terreus* VKM Y-2253 as the test culture. The activity was observed within the pH range from 3.5 to 5.0 and reached the maximum for both isolates at pH 4.0–4.5 on both GPA and MA independently of the buffer (citrate–phosphate or sodium succinate) applied. The diameter of the growth inhibition zones increased with a decrease in the concentration of the nitrogen source (peptone or $(NH_4)_2SO_4$) and with an increase in glucose content of GPA. The antifungal agent was also produced by strains cultivated in liquid media both under static conditions and on a shaker (150 rpm).

The antifungal agent secreted by *P. graminicola* was characterized by an extremely broad spectrum of action. Almost all of the tested 377 strains of ascomycetous and basidiomycetous fungi belonging to 268 species of 95 genera were sensitive to this agent (Table 1). Resistant species were represented by *Bullera hannae*, *Cryptococcus nemorosus*, *C. perniciosus*, *Dacrymyces stillatus*, *Neovossia setariae*, and *Sporobolomyces singularis*, as well as by several strains of *C. humicola* and *Ustilago maydis*.

To date, yeasts are known to secret two types of antifungal agents, viz., mycocins and glycolipids [2]. The former are of a proteinaceous nature and, unlike the latter, are characterized by taxon-specific action. The antifungal agent of *P. graminicola* was active against almost all ascomycetous and basidiomycetous fungi tested (Table 1); this is indicative of its glycolipid nature. This assumption is confirmed by the solubility of this agent in methanol and its insolubility in water.

When strains L1-20 and L1-46 were incubated at the maximum growth temperature (35°C) under conditions of effective elimination of extrachromosomal genetic elements, two types of colonies were formed: those with even and rhizoidal edges. The colonies of the latter type were characterized by more intensive mycelium formation than those with entire edges. More than 70 colonies of both types were tested; all of them retained their antifungal activity, although the size of the growth inhibition zones varied. The absence of eliminants is indicative of the chromosome localization of the *P. graminicola* genes encoding the synthesis of the antifungal agent, whereas the mycocin synthesis is often associated with the presence of viruses or plasmids in the cells [2].

The TLC analysis of the purified antifungal agent secreted by *P. graminicola* revealed one spot with R_f 0.75. Under the cultivation conditions applied, the yield of the antifungal agent was 20–25 mg/l. Methanol solutions of the preparation stored at 4°C retained their activity for at least 1.5 years.

The TLC analysis of the antifungal hydrolysate subjected to methanolysis revealed two additional spots, of which one had the R_f value similar to that of glucose; the other spot was identified as methyl ether of C_{16}-tri-

hydroxy acid on the basis of its molecular mass (341 Da). According to the data of mass spectrometry, the molecular mass of the compound secreted by *P. graminicola* was 783.8 Da.

The ^{13}C-NMR spectrum of the purified preparation contained two signals in the resonance region of anomeric carbon atoms of the carbohydrate residues (δ_C 105.0 and 102.2), three signals of CO groups (δ_C 179.6, 172.3, and 171.25), signals of CH_3CO (δ_C 20.9) and CH_3-C (δ_C 14.4) groups, signals of different intensity corresponding to C-CH_2-C group (δ_C 43.7–19.5), and two signals of O-CH_2-C groups (δ_C 64.2 and 62.2). Other signals of O-CH-C groups were at 68.3–81.95 ppm. The ^{1}H-NMR spectrum contained two pairs of signals typical of anomeric carbohydrate protons (δ_H 5.21 and 4.89, $^3J_{1,2}$ 8 Hz), signals of CH_2CO groups (δ_H 3.01 and 2.99), protons of CH_2-CH_3 groups (triplet at δ_H 0.90, ^{3}J 6 Hz), and the CH_3CO group (singlet at δ_H 2.07). All these spectral characteristics are completely identical to those of the cellobiose lipid secreted by *P. fusiformata*, another *Pseudozyma* species [5]. According to the results of two-dimensional spectroscopy (2D COSY and TOCSY), the preparation produced by *P. graminicola* contained two residues of β-glucopyranose, the residue of 2,15,16-trihydroxyhexadecanoic acid, and the residue of 3-hydroxyhexanoic acid. The 2D ROESY spectrum showed the linkage between the anomeric proton (δ_H 5.21) and the H-4 proton of the other β-glucopyranose residue (δ_H 4.07), indicating the presence of a β, 1 ⇢ 4 bond between them. The signal of the second anomeric proton (δ_H 4.89) was related to H-16 of 2,15,16-trihydroxyhexadecanoic acid (peak correlations δ_H/δ_H were equal to 4.07/4.20 and 4.07/3.88); therefore, the residue of 4-O-β-D-glucopyranosil-D-glucose (cellobiose) has a glycoside bond with the 16th carbon atom of the acid residue. In addition to the data of ^{13}C-NMR, the analysis of the [^{1}H, ^{13}C]-HSQC spectrum confirmed the substitution of C-4' and C-16 (δ_C 81.95 and 75.9), whereas the HMBC spectrum revealed a correlation between the following peaks: H-1"/C-4' (δ_H/δ_C 5.21/81.95) and H-1'/C-16 (δ_H/δ_C 4.89/75.9); CH_3CO/CH_3CO (δ_H/δ_C 2.03/171.25) and H-6'/CH_3CO (δ_H/δ_C 4.85/171.25 and 4.64/171.25); H-2"/C-1'" (δ_H/δ_C 5.66/172.3), and H-2'"/C-1'" (δ_H/δ_C 3.01/172.3 and δ_H/δ_C 2.99/172.3). The first two correlations confirm positions of the residues of β-glucopyranose and 2,15,16-trihydroxyhexadecanoic acid and their substituents. The other correlation peaks revealed the location of O-acetyl groups in the position 6' of the internal residue of β-glucopyranose and the residue of 3-hydroxyhexanoic acid located in C-2" position of the terminal residue of β-glucopyranose. The location of signals belonging to H-2" (δ_H 5.66) and H-6' (δ_H 4.85; 4.64) is consistent with the known effect of O-acetylation on the ^{1}-NMR spectra of carbohydrates.

Table 1. The fungal genera sensitive to the extracellular cellobiose lipid produced by strains *Pseudozyma graminicola* L1-20 and L1-46

Ambrosiozyma (4, 4)	*Nakazawaea* (1, 1)
Arthroascus (2, 2)	*Naumovia* (1, 1)
Bensingtonia (8, 8)	*Nematospora* (1, 1)
Bullera (5, 5)*	*Pachysolen* (1, 1)
Bulleromyces (1, 1)	*Phomopsis* (1, 1)
Candida (8, 9)	*Pichia* (4, 4)
Citeromyces (1, 1)	*Protomyces* (3, 3)
Clavispora (1, 1)	*Pseudozyma* (5, 11)
Cryptococcus (39, 54)*	*Puccinia* (3, 3)*
Curvibasidium (1, 1)	*Rhodosporidium* (8, 15)
Cystofilobasidium (3, 4)	*Rhodotorula* (16, 44)
Debaryomyces (10, 12)	*Saccharomyces* (5, 6)
Dekkera (1, 1)	*Saccharomycodes* (1, 4)
Dioszegia (2, 2)	*Saccharomycopsis* (1, 1)
Entyloma (1, 1)	*Sakaguchia* (1, 2)
Erythrobasidium (1, 1)	*Saturnispora* (1, 1)
Exobasidium (6, 8)	*Schizosaccharomyces* (2, 4)
Farysia (1, 1)	*Schwanniomyces* (1, 1)
Fellomyces (1, 1)	*Sclerotinia* (1, 1)
Fibulobasidium (1, 1)	*Sirobasidium* (1, 1)
Filobasidiella (1, 2)	*Sphacelotheca* (1, 1)
Filobasidium (1, 1)	*Sporidiobolus* (5, 6)
Geotrichum (1, 1)	*Sporisorium* (1, 1)
Guehomyces (1, 2)	*Sporobolomyces* (3, 3)*
Guillermondella (1, 1)	*Sterigmatomyces* (2, 3)
Hanseniaspora (2, 2)	*Sterigmatosporidium* (1, 3)
Holtermannia (1, 1)	*Sympodiomycopsis* (1, 1)
Itersonilia (1, 1)	*Taphrina* (7, 7)
Kazachstania (6, 6)	*Tetrapisispora* (1, 1)
Kluyveromyces (3, 5)	*Tilletia* (1, 1)
Kockovaella (1, 1)	*Tilletiopsis* (3, 4)
Kodamea (1, 1)	*Torulaspora* (3, 3)
Komagataea (1, 1)	*Tremella* (2, 2)
Komagataella (1, 1)	*Trichosporon* (9, 9)
Kondoa (1, 1)	*Udeniomyces* (1, 1)
Kurtzmanomyces (1, 1)	*Ustilago* (6, 7)*
Lachancea (1, 1)	*Vanderwaltozyma* (1, 1)
Leucosporidium (2, 2)	*Wickerhamia* (1, 1)
Lipomyces (2, 2)	*Wickerhamiella* (1, 1)
Lodderomyces (1, 1)	*Williopsis* (1, 1)
Mastigobasidium (1, 1)	*Xanthophyllomyces* (1, 2)
Mastigomyces (2, 2)	*Yarrowia* (1, 9)
Metschnikowia (8, 22)	*Zygoascus* (1, 2)
Microbotryum (5, 7)	*Zygosaccharomyces* (1, 2)
Mrakia (2, 3)	*Zygotorulaspora* (1, 1)
Nadsonia (2, 3)	*Zygowilliopsis* (1, 1)
Nakaseomyces (1, 1)	

Note: * Resistant species or strains were also revealed (see text), but not included in the Table. Figures in parentheses show the number of tested species and strains, respectively.

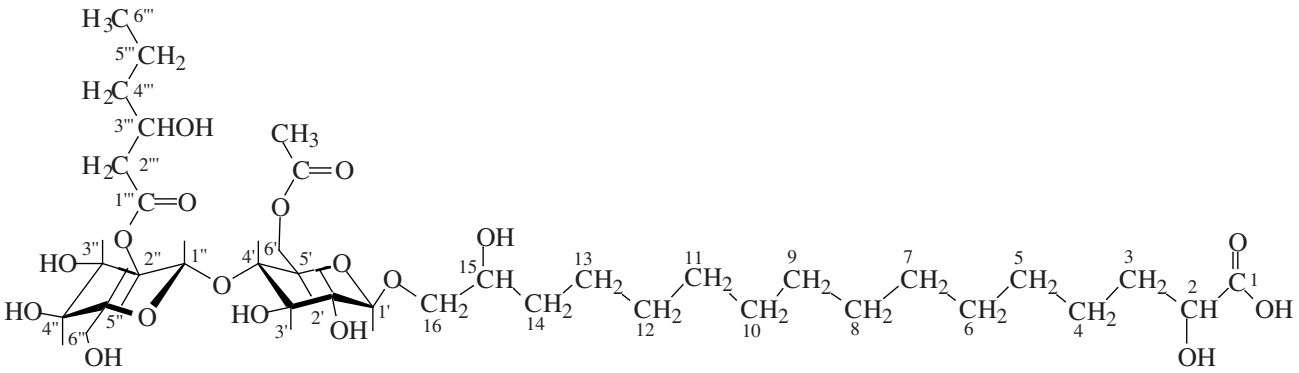

Fig. 1. The structure of the extracellular cellobiose lipid produced by *Pseudozyma graminicola.*

Based on the results obtained by NMR spectroscopy, mass spectrometry, and methanolysis, it can be assumed that antifungal agent produced by *P. graminicola* represents a cellobiose lipid containing 2,15,16-trihydroxypalmitic acid as an aglycon and cellobiose residue with O-substituents, viz., a 3-hydroxycaproic acid at the terminal glucose residue and an acetyl group at the internal residue (Fig. 1). The same product was secreted by other species of smut fungi of the family *Ustilaginaceae*, such as *P. flocculosa* [9], *P. fusiformata* [5], and *U. maydis* [10]. Similar cellobiose lipids were synthesized by representatives of the orders *Microstromatales* [11] and *Trichosporonales* [12]; however, they differed either in the lack of O-substituents or in the level of cellobiose acetylation.

The cellobiose lipid of *P. graminicola* exhibited antifungal activity against the yeastlike fungi pathogenic to humans (causing candidosis and cryptococcosis). This activity was most pronounced against basidiomycetous yeasts, which were killed after a 30-min incubation with the antifungal agent at concentrations of 0.01–0.05 mg/ml, whereas in the case of asomycetous yeasts, such an effect was observed at concentrations of the agent higher by an order of magnitude (Table 2). The cellobiose lipid secreted by *P. graminicola* caused the release of ATP from yeast cells, which was at its most intensive at a pH about 4.5 (Fig. 2); at

Table 2. The survival of ascomycetous and basidiomycetous yeasts after a 30-min incubation with the cellobiose lipid of strain *Pseudozyma graminicola* L1-46 (initial yeast concentration was 5×10^6 cells/ml; pH 4.0; 0.04 M citrate–phosphate buffer, 25°C)

Species and strains	The number of survived cells (% of control)* at the cellobiose lipid concentrations (mg/ml)							
	0	0.01	0.02	0.05	0.10	0.20	0.30	0.50
Ascomycetes								
Candida albicans JCM 1542	100	100	–**	–	21	2	1	0
C. glabrata CBS 138	100	100	–	–	43	30	23	2
C. viswanathii CBS 4024	100	46	40	29	3	1	–	–
Clavispora lusitaniae IGC 2705	100	100	–	–	4	1	1	0
Saccharomyces cerevisiae VKM Y-1173	100	100	–	–	14	5	3	0
Basidiomycetes								
Cryptococcus terreus VKM Y-2253	100	4	2	0	0	0	–	–
Filobasidiella neoformans IGC 3957	100	27	16	4	0	0	–	–

Notes: CBS: Centraalbureau voor Schimmelcultures, the Netherlands; IGC: Instituto Gulbenkian de Ciencia, Portugal; JCM Collection of Microorganisms, Japan.

* Data are means of triplicate determinations.

** Not detected.

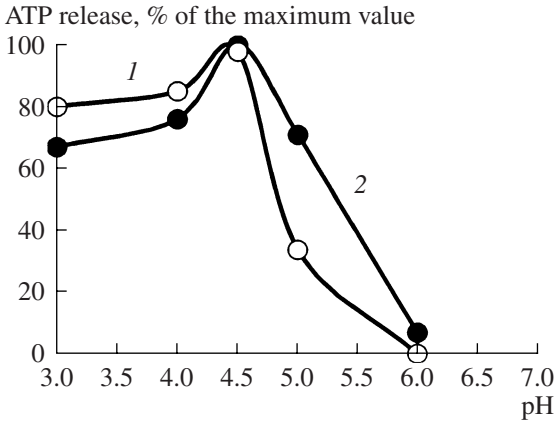

Fig. 2. ATP release from the cells of *Cryptococcus terreus* VKM Y-2253 after 30-min treatment with the cellobiose lipid (0.16 mg/ml) of strains *Pseudozyma graminicola* L1-20 (*1*) and L1-46 (*2*) at different pH values (0.04 M citrate–phosphate buffer) at 20°C.

this pH value, the largest growth inhibition zones of the test fungi were observed on GPA by the culture-to-culture method. The ATP release indicated an increase in the permeability as a result of the membrane-damaging activity of the cellobiose lipid. The maximum ATP release from the cells of basidiomycetous yeast *C. terreus* VKM Y-2253 and ascosporic yeast *S. cerevisiae* VKM Y-1173 was observed at concentrations of the cellobiose lipid of 0.06 and 0.3 mg/l, respectively. Such differences in the cell sensitivity between asco- and basidiomycetous fungi appeared to be associated with the different composition of their membranes [2, 13].

Secretion of antifungal substances is primarily aimed at increasing the competitive ability of the producers. At the same time, antifungal activity of epiphytic yeasts appeared to be a part of the natural system of plant protection against pathogens. The presence of a hydroxy fatty acid in molecules of cellobiose lipid is arguably not accidental, since the synthesis of these acids is known to be a defense reaction of plants against fungal invasion [14]. It is assumed that C_{16}- and C_{18}-hydroxy acids act either directly as fungicides or as signal molecules triggering the mechanisms of plant protection. Cellobiose lipids of yeasts as natural antifungal agents are considered to be a promising alternative to toxic synthetic fungicides.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (projects 06-04-48215-a and 06-04-08253-ofi).

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