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Ustilagic acid secretion by *Pseudozyma fusiformata* strains

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Abstract

Eight strains of *Pseudozyma fusiformata* were examined for antifungal activity. All of them had the same spectrum of action and were active against many species of yeasts, yeast-like and filamentous fungi. They secreted glycolipids, which were purified from the culture liquid by column and thin-layer chromatography. According to nuclear magnetic resonance and mass-spectroscopy experiments all strains produced ustilagic acid, a cellobioside-containing 2,15,16-trihydroxypalmitic acid as aglycon, 3-hydroxycaproic acid and acetic acid as *O*-acylic substituents.
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1. Introduction

Many yeasts are members of the microbial biota associated with plants. Traditionally, they have been considered mainly as suppliers of growth-promoting substances for other members of biocenoses [1]. However, the discovery of their antifungal activity stemming from secretion of mycocins [2,3] or glycolipids [4–8] has changed our understanding of their role in communities of fungi. The identification of glycolipids produced by different yeast species raised new challenges in the search for potential fungicidal agents and the elucidation of their mode of action.

Recently, we have observed that the ustilaginaceous yeast species *Pseudozyma fusiformata* (Buhagiar) Boekhout displays antimycotic activity against many yeasts and filamentous fungi, including phytopathogenic and medically important species of the genera *Filobasidiella*, *Malassezia*, *Taphrina* and *Ustilago* [4]. An extracellular agent consisting of glucose and fatty acids apparently caused this activity [4]. It enhanced the cytoplasmic membrane permeability, leading to leakage of intracellular compounds [5].

This work aimed at the elucidation of the structures of antifungal glycolipids secreted by different isolates of *Ps. fusiformata*.

2. Materials and methods

2.1. Strains and culture conditions

The *Ps. fusiformata* strains examined are listed in Table 1. With the exception of *Candida albicans* JCM 1542, all other strains were obtained from the Russian Collection of Microorganisms (VKM, http://www.vkm.ru).

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Table 1
List of *Pseudozyma fusiformata* strains examined

Strain number	Source, date of isolation	Locality
VKM Y-2821	Cauliflower, 1975	Warwickshire, UK
VKM Y-2898	Grass leaves, 1999	Prioksko-terrasny biosphere reserve, Moscow region, Russia
VKM Y-2909	Forest litter, 2000	Prioksko-terrasny biosphere reserve, Moscow region, Russia
Ll-16	Grass leaves, 2002	Moscow region, Russia
Ll-41	Grass leaves, 2002	Moscow region, Russia
Ll-71	Grass leaves, 2002	Moscow region, Russia
PTZ-351	Grass leaves, 1999	Prioksko-terrasny biosphere reserve, Moscow region, Russia
PTZ-356	Grass leaves, 1999	Prioksko-terrasny biosphere reserve, Moscow region, Russia

The glycolipid-producing strains were grown without shaking at 24 °C for 1 month in a liquid medium. The medium contained: 1.0% glucose, 0.1% $(NH_4)_2SO_4$, 0.005% $MgSO_4$, 0.05% yeast extract and 0.73% citric acid. The pH was adjusted by Na_2HPO_4 to 4.0. During the cultivation no contamination was observed by light microscopy.

The growth conditions of the strains used for the assay of antifungal activity have been described in [5].

2.2. Purification of glycolipid

The filtrates of culture liquid (3 l) were lyophilised and extracted with 500 ml methanol. Filtered extracts were evaporated to 100 ml, the salt sediments were removed by filtration and the supernatants were evaporated to almost dryness. The substance obtained was resolved in 250 ml cold deionized water and the suspension was kept at 4 °C for 24 h. The insoluble compounds were separated by filtration, washed twice with cold water and resolved in methanol. Then the glycolipids were purified by chromatography [5]. The antifungal activity during the purification steps was assayed by measuring the diameter of growth inhibition zones of *Cryptococcus terreus* VKM Y-2253 [5]. The measurement of ATP leakage from yeast cells treated with glycolipid preparations was performed by the luciferine–luciferase method [5]. After evaporation of methanol, the concentration of glycolipid was determined by weighing.

2.3. NMR spectroscopy and ESI-MS analysis

One-dimensional ^{1}H NMR, ^{13}C NMR, two-dimensional ^{1}H,^{1}H COSY, TOCSY, ROESY, [^{1}H,^{13}C] HSQC and [^{1}H,^{13}C] HMBC spectra were recorded with a Bruker DRX-500 spectrometer (Bruker, Rheinstetten, Germany) at 30 °C. The sample was examined in pyridine-d_5 with TMS as an internal chemical-shift reference. 2D-spectra were obtained using standard pulse sequences from the Bruker software (XWINNMR 1.2). A mixing time of 200 and 100 ms was used in 2D TOCSY and ROESY experiments, respectively. The two dimensional HMBC spectrum was optimized for the coupling constant $J_{H,C}$ of 5 Hz.

The glycolipids were also analyzed by electrospray mass spectrometry in the positive mode using an LCO DECAXP mass spectrometer (Thermo Finnigan, San José, CA, USA). The samples were solved in methanol and sprayed at a flow rate of 3 μl min^{-1}.

3. Results

All *Ps. fusiformata* strains examined have antimycotic activity against many ascomycetous and basidiomycetous yeasts and filamentous fungi, and generally their action spectra do not differ from the spectrum described for strain VKM Y-2821 [4]. Similar to the latter (Fig. 1) the preparations obtained from the other strains under study induced ATP leakage from yeast cells.

The preparation of *Ps. fusiformata* VKM Y-2821 was studied by ^{1}H- and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, ROESY, [^{1}H,^{13}C] HSQC and [^{1}H,^{13}C] HMBC experiments. The ^{13}C NMR spectrum of the purified glycolipid contained two signals for anomeric carbon atoms of sugar residues (δ_C 105.0 and 102.2 ppm), three signals of CO groups (δ_C 179.6,

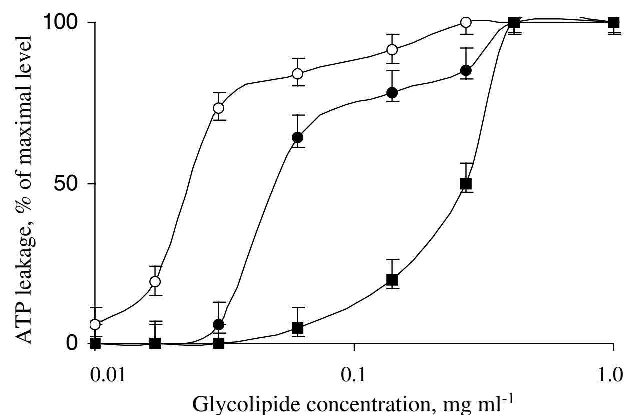

Fig. 1. ATP leakage from the cells of *Cryptococcus terreus* VKM Y-2253 (○), *Candida albicans* JCM 1542 (●) and *Saccharomyces cerevisiae* VKM-1173 (■) treated by purified glycolipids of *Pseudozyma fusiformata* VKM Y-2821. The cells were incubated with different concentrations of glycolipids in 0.04-M citrate-phosphate buffer, pH 4.0, at 20 °C for 30 min. The mean values with standard deviations are shown.

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172.3 and 171.25 ppm), signals for CH_3CO (δ_C 20.9 ppm) and CH_3–C (δ_C 14.4 ppm), signals C–CH_2–C of a different intensity (δ_C 43.7–19.5 ppm), and two signals of O–CH_2–C groups (δ_C 64.2 and 62.5 ppm). The other signals for O–*C*H–C groups were located in the region 68.3–81.95 ppm.

The ^{1}H NMR spectrum contained inter alia two doublets characteristic of anomeric sugar protons (δ_H 5.21 and 4.89 ppm, $^3J_{1,2}$ 8 Hz), an AB spin system of a CH_2CO group (δ_H 3.01 and 2.99 ppm), protons of a $–CH_2–CH_3$ group (triplet at δ_H 0.90, ^3J 6 Hz) and a CH_3CO group (singlet at δ_H 2.07 ppm). The spectrum was assigned using 2D COSY and TOCSY experiments (Table 2). Analysis of the 2D spectra revealed two residues of β-glucopyranoses, a residue of 2,15,16-trihydroxy-palmitic acid, containing 16 carbon atoms, and a residue of 3-hydroxycaproic acid, containing six carbon atoms.

The 2D ROESY spectrum showed spatial contact of the anomeric proton at δ_H 5.21 with proton H-4 of the other β-glucopyranose residue (δ_H 4.07 ppm), demonstrating the presence of a β,1 → 4 linkage between two residues. The second anomeric proton at δ_H 4.89 proved to be close to H-16 of the 2,15,16-trihydroxy-palmitic acid (correlation peaks δ_H/δ_H 4.07/4.20 and 4.07/3.88). Thus, the cellobiose residue was linked to C-16 of the 2,15,16-trihydroxy-palmitic acid by a glycosidic linkage.

The assignment in the ^{13}C NMR spectrum (Table 2) was done on the basis of ^{1}H,^{13}C HSQC and HMBC experiments. The assignment in the HSQC spectrum confirmed the substitution of C-4′ and C-16 (Fig. 2) due to relatively low-field position of the corresponding signals (δ_C 81.95 and 75.9 ppm). The HMBC spectrum contained inter alia the following inter- and intra-residue correlation peaks: H-1″/C-4′ (δ_H/δ_C 5.21/81.95), 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 confirmed the sequence of β-glucopyranose residues and the residue of 2,15,16-trihydroxy-palmitic acid, as well as positions of the substitution in the residues. Other correlation peaks revealed the location of an *O*-acetyl group at position 6′ of the inner β-glucopyranose residue and a 3-hydroxycaproic acid residue at C-2″ of the terminal β-glucopyranose residue. The relatively low-field positions of the H-2″

Table 2
125-MHz ^{13}C NMR and 500-MHz ^{1}H NMR chemical shifts of the cellobiose glycolipid of *Ps. fusiformata* (solution in pyridine-d_5, internal TMS as reference)

Carbon atom	δ_C	δ_H[a]
C-1	179.6	
C-2	72.3	4.67
C-3	35.9	2.21; 2.09
C-4	26.3	1.79
C-5	30.2	1.38
C-(6-11)	30.15	1.18–1.24
C-12	30.2	1.28
C-13	26.3	1.65; 1.49
C-14	34.4	1.64
C-15	70.8	4.15
C-16	75.9	4.20; 3.88
C-1′	105.0	4.89
C-2′	74.7	4.07
C-3′	76.3	4.26
C-4′	81.95	4.07
C-5′	73.15	4.00
C-6′	64.2	4.85; 4.64
C-1″	102.2	5.21
C-2″	75.4	5.66
C-3″	76.3	4.26
C-4″	71.9	4.12
C-5″	78.8	4.09
C-6″	62.5	4.60; 4.21
C-1‴	172.3	
C-2‴	43.7	3.01; 2.99
C-3‴	68.2	4.54
C-4‴	40.3	1.72
C-5‴	19.5	1.69; 1.55
C-6‴	14.4	0.90
CH_3CO	20.7; 171.25	2.03

[a] Chemical shifts for the corresponding attached proton(s).

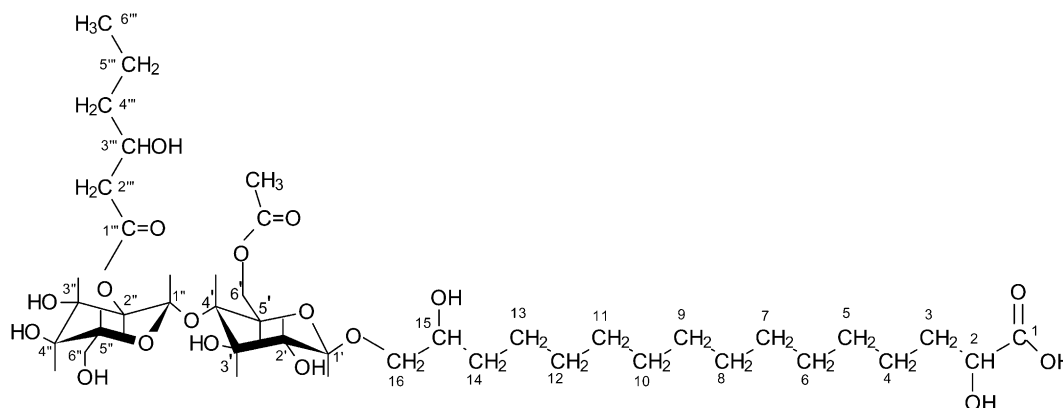

Fig. 2. The structure of the antifungal glycolipid (784.8 Da) of *Pseudozyma fusiformata*.

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(δ_H 5.66 ppm) and H-6′ (δ_H 4.85; 4.64 ppm) were in agreement with well-known effects of *O*-acylation in the ^{1}H NMR spectra of carbohydrates. Thus, both ^{1}H- and ^{13}C NMR data led to the formula (Fig. 2) for the glycolipid under investigation.

The ESI analysis of the glycolipid preparation of *Ps. fusiformata* VKM Y-2821 in positive mode gave a major signal at 807.7 Da, corresponding to a molecular mass of 784.8, plus 22.9 Da for sodium. This value is in agreement with the chemical structure depicted in Fig. 2.

The preparations obtained from the strains VKM Y-2909, Ll-16, Ll-71, and PTZ-351 contained the glycolipid of 784.8 Da only. In the mass spectra of preparations obtained from the strains VKM Y-2821, VKM Y-2898, PTZ-356, and Ll-41, an additional signal was observed at 693.7 Da, corresponding to a molecular mass of 670.7, plus 22.9 for sodium. This signal indicates the presence of a glycolipid without a 3-hydroxycaproic acid residue (114 Da) in 2″ position. Its content in the preparations varied from 30% to 60% according to mass spectrometry.

4. Discussion

The results of our structural analyses disclosed that, regardless of their origin (Table 1), all *Ps. fusiformata* strains secrete the same glycolipids. Most likely they only produce the cellobiose lipid depicted in Fig. 2. As the presence of the glycolipid of 670.7 Da in some preparations was probably due to splitting-off of the 3-hydroxycaproic acid residue, caused by lability of the ester bond. The absence of any essential difference between the action spectra of the *Ps. fusiformata* strains is in agreement with this hypothesis.

A partly acylated cellobioside of 2,15,16-trihydroxypalmitic acid and 3-hydroxycaproic acid has been known for a long time in *Ustilago maydis* (de Candolle) Corda under the name ustilagic acid [9,10]. *U. maydis* and *Ps. fusiformata* are related to each other phylogenetically and belong to sister clades in the Ustilaginales (Ustilaginomycetidae, Ustilaginomycetes) [11,12]. The identity of their extracellular glycolipids may be additional evidence of their phylogenetic relationship. The species *Ps. flocculosa* is also closely related to *U. maydis*, but its glycolipid has been identified as 2-(2′,4′-diacetoxy-5′-carboxy-pentanoyl)-octadecyl-cellobioside [6]. The significance of the structural differences of the *Ps. flocculosa* glycolipid from ustilagic acid needs further investigation. Another ustilaginaceous yeast, *Sympodiomycopsis paphiopedili* Sugiyama et al. (Microstromatales, Exobasidiomycetidae, Ustilaginomycetes), is distantly related to *U. maydis*, but it secretes a similar cellobiose lipid that differs from ustilagic acid by the absence of 3-hydroxycaproic acid and acetate residues as *O*-acylic substituents [8]. Probably, the cellobioside of 2,15,16-trihydroxypalmitic acid is a basic unit of antimycotic glycolipids produced by smut fungi and the presence of the 3-hydroxycaproic acid residue has no effect on the antifungal activity.

The cellobiose lipids of *Ps. fusiformata* and *Sym. paphiopedili* have broad ranges of action. They were found active against almost all strains of yeasts, yeast-like and filamentous fungi tested among 300 species belonging to both the *Ascomycota* and *Basidiomycota* [4,7]. We are inclined to postulate that the majority, if not all, *Ascomycota* and *Basidiomycota* are sensitive to these cellobiose lipids, but at different inhibitory concentrations. For example, the minimal effective concentration of the *Ps. fusiformata* glycolipid leading to ATP leakage for *Cr. terreus* di Menna was 0.03 mg ml^{-1}, whereas for *Saccharomyces cerevisiae* Meyen *ex* Hansen this value was much higher, namely 0.4 mg ml^{-1} (Fig. 1). Probably, the cellobiose lipids of *Ps. flocculosa* and *U. maydis* also have broad action spectra, but so far they have only been tested against a limited number of fungi [6,13], while the bioassay of their activity was performed under non-optimal conditions [9,10], as fungicidal activity of cellobiose lipids is expressed at acidic pH values [5,8].

The broad spectra of activity, pH and temperature stability allow to consider cellobiose lipids as promising natural biocontrol agents of pathogenic fungi, and to prevent fungal spoilage of feed and food [14].

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