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EXPERIMENTAL ARTICLES

The Fungicidal Activity of an Extracellular Glycolipid from *Sympodiomycopsis paphiopedili* Sugiyama *et al.*

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Abstract—The yeast *Sympodiomycopsis paphiopedili* (*Ustilaginomycetes*) produces an extracellular glycolipid, which possesses the maximum antifungal activity at a pH of the medium equal to 4.0–4.5. Among the approximately 300 tested species of yeastlike and mycelial fungi, more than 80% (including species pathogenic for plants, animals, and humans) were found to be sensitive to this glycolipid.

Key words: glycolipid, fungicide, antagonism, yeasts.

The production of extracellular glycolipids by the yeastlike and mycelial fungi of the genera *Candida, Rhodotorula, Shizonella, Ustilago*, and *Yarrowia* has long been known. Functionally, these glycolipids are usually considered to be surfactants providing for the absorption of hydrophobic compounds by microbial cells [1–3]. However, recent studies showed that the extracellular glycolipids of *Pseudozyma fusiformata* [4], *Ps. flocculosa* [5], and *Cryptococcus humicola* [6] possess fungicidal activity against a wide range of yeastlike fungi, including ones pathogenic for plants, animals, and humans. This activity is maximum at acidic pH values of the medium and is due to changes in the permeability of the cytoplasmic membrane induced by the glycolipids [6, 7].

The study of antagonistic interactions between smut fungi [4, 8, 9] allowed us to reveal the antifungal activity of *Sympodiomycopsis paphiopedili*, a species of the genus *Sympodiomycopsis* Sugiyama *et al.* [10].

The aim of this work was to study the conditions under which this activity is maximum and to investigate the nature of the fungicidal agent and the range of sensitive organisms.

MATERIALS AND METHODS

The strains used in the study were obtained from the All-Russia Collection of Microorganisms (VKM, http://www.vkm.ru); the Japan Collection of Microorganisms (JCM, http://www.jcm.riken.go.jp); the collection at the Department of Microbiology, Pharmaceutical College, Tanashi, Japan; and the collection at the Institute of Oil-Producing Plants, Krasnodar, Russia. The sensitivity of these strains to the extracellular agent of *Sympodiomycopsis paphiopedili* VKM Y-2817 was tested by the culture-to-culture method (Fig. 1) on glucose–peptone agar (GPA) plates at 17°C. GPA contained 0.5% glucose, 0.25% peptone, 0.2% yeast extract, and 50 ml/l glycerol in sodium succinate buffer (pH 4.0).

Fig. 1. The inhibition of the growth of the white rot fungus *Sclerotinia sclerotiorum* by *Symp. paphiopedili* VKM Y-2817 on GPA plates (pH 4.0) with 0.003% methylene blue.

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In testing yeasts from the genus *Malassezia*, the medium was supplemented with olive oil (10 ml/l).

To prepare the fungicidal agent, strain VKM Y-2817 was grown at room temperature (20–24°C) for four weeks in a medium containing (%) glucose, 1; $(NH_4)_2SO_4$, 0.1; yeast extract, 0.05; and $MgSO_4$, 0.005 in sodium succinate buffer (pH 4.0). Cells were removed by centrifugation at 5000 *g* for 30 min at 0°C. The supernatant was passed through a GF/A filter (Whatman) and lyophilized. The powder was extracted with methanol for 24 h. The extract was evaporated at 40–50°C nearly to dryness. The residue was diluted with 200 ml of deionized water, and the solution was kept at 4°C overnight. The precipitate was harvested by centrifugation at 4000 *g* for 15 min at 0°C, washed twice with water, and dissolved in methanol. The solution was applied onto a (25 × 1.5 cm) column packed with Sephadex LH (Pharmacia, Sweden). The column was washed with methanol at a flow rate of 15 ml/h. The fractions (4 ml) were tested for antifungal activity as follows: Aliquots (10 μl) were transferred to GF/A filters (5 mm in diameter) and dried. The dry filters were placed onto GPA (pH 4.0) plates with a lawn of *Cryptococcus terreus* VKM Y-2253 cells. The fractions that exhibited the maximum antifungal activity were pooled and subjected to further purification on HP-KF silica gel plates (Whatman) developed in a chloroform–methanol–water (4 : 4 : 0.2) solvent system. To visualize the antifungal agent, one plate was sprayed with a 5% solution of H_2SO_4 in ethanol and dried at 100°C. The corresponding zones of the other plates were extracted with methanol. The extract was stored at 4°C for future use.

To determine the amino acid and sugar compositions of the antifungal agent, the extract was dried in a flow of nitrogen. The residue was hydrolyzed at 100°C in 6 N HCl for 6 h (for amino acid analysis) or in 3 M HF for 0.5, 3, and 6 h (for sugar analysis). The hydrolysates (as well as the preparation before hydrolysis) were analyzed with an AAA-881 amino acid analyzer (Microtechna, Czech Republic) and a sugar analyzer (Biotronic, Germany). The ^{1}H NMR spectrum of the antifungal preparation was recorded in deuterated methanol with a WH-400 spectrometer (Bruker, Germany).

The viability of yeast cells after their incubation with the antifungal agent was determined by plating cell suspensions on malt extract agar.

To cure strain VKM Y-2817 of its antibiotic phenotype, the strain was incubated on malt extract agar at 30°C (the maximum growth temperature of this strain is 32°C).

RESULTS

The antifungal activity of *Symp. paphiopedili* VKM Y-2817 was observed between pH 3.5 and 5.0, being maximum at pH 4.0–4.5. At pH 5.5, antifungal activity was absent. The incubation of strain VKM Y-2817 at 30°C did not eliminate its antifungal phenotype, as is evident from the fact that none of the approximately 300 randomly chosen colonies of this strain lost antifungal activity after the treatment.

Among the 464 tested strains of yeastlike and mycelial fungi, belonging to 301 species of 86 genera, 84% were found to be sensitive to the antifungal agent of strain VKM Y-2817 (table). On the other hand, 11 genera turned out to be resistant to the antifungal agent, namely, *Ambroziozyma* (4, 4), *Atractogloea* (1, 1), *Dacrymyces* (1, 1), *Entyloma* (1, 1), *Guepiniopsis* (1, 1), *Hanseniaspora* (3, 4), *Neovossia* (2, 2), *Platygloea* (1, 1), *Saccharomyces* (14, 18), *Saccharomycodes* (1, 6), and *Yarrowia* (1, 9). (Here and below, figures in parentheses show the number of species and strains tested.) As a rule, all species of a particular tested genus were either sensitive or resistant to the antifungal agent of strain VKM Y-2817. However, the essentially sensitive genera *Exobasidium*, *Kluyveromyces, Pseudozyma, Schizosaccharomyces, Taphrina, Tilletiopsis*, and *Ustilago* were heterogeneous in this respect. For instance, the species *Ex. vaccinii* (2), *Ex. pachysporum* (1), *K. africanus* (1), *K. lactis* (2), *Ps. aphidis* (1), *Ps. prolifica* (1), *Rh. laryngis* (1), *Schiz. pombe* (11), *T. caerulescens* (1), *T. carnea* (1), *T. populi-salicis* (1), *T. purpurescens* (1), *T. sadebeckii* (1), *Til. albescens* (2), *U. cynodontis* (2), and *U. maydis* (5) of these genera were found to be resistant to the antifungal agent. Conversely, two species, *Sacch. kluyveri* (1) and *Sacch. kunashiriensis* (1), of the mainly resistant genus *Saccharomyces* (18, 22) were slightly sensitive to the antifungal agent of strain VKM Y-2817.

After chromatography on Sephadex LH, antifungal activity was mainly found in the fraction eluted after actinomycin D (molecular mass, 1255 Da). Consequently, the antifungal agent has a molecular mass of less than 1250. The thin-layer chromatography (TLC) of this fraction in the chloroform–methanol–water (4 : 4 : 0.2) system revealed several spots, one of which (with R_f = 0.75) possessed antifungal activity. The repeated TLC of the material from this spot in the solvent system with varied proportions of the components gave rise to a single spot. The yield of this substance was 20–50 mg per liter of the culture liquid.

The purified preparation was fairly soluble in methanol and pyridine, poorly soluble in chloroform, and nearly insoluble in water. The preparation did not react with ninhydrin and showed the presence of amino acids neither before nor after acid hydrolysis. The ^{1}H NMR spectrum of the substance contained resonance signals at 1.7–0.9, 2.0, 3.0–3.9, and 4.2–4.5 ppm, which corresponded to the protons of the functional groups of fatty acids and sugars ($–CH_2–$, $–CH_3CO–$, pyranose cycles, –OH, and –CH=CH–) (Fig. 2). The acid hydrolysates of the preparation contained glucose and cellobiose. The concentration of glucose increased and that of cellobiose decreased when the time of hydrolysis was extended. After 6 h of hydrolysis, the concentration of

Fungal genera sensitive to the extracellular agent of *Symp. paphiopedili* VKM Y-2817

Agaricostilbum (1, 1)	*Gymnosporangium* (1, 1)	*Schizosaccharomyces* (2, 6)*
Arthroascus (1, 1)	*Holtermannia* (1, 1)	*Sclerotinia* (1, 1)
Arxula (1, 1)	*Issatchenkia* (1, 1)	*Septobasidium* (1, 1)
Bensingtonia (6, 6)	*Itersonilia* (1, 2)	*Sorosporium* (1, 1)
Bullera (11, 28)	*Kluyveromyces* (4, 5)	*Sphacelotheca* (1, 1)
Bulleromyces (1, 1)	*Kockovaella* (1, 1)	*Sporidiobolus* (4, 4)
Candida (3, 3)	*Kurtzmanomyces* (1, 1)	*Sporisorium* (2, 3)
Citeromyces (1, 1)	*Leucosporidium* (2, 2)	*Sporobolomyces* (24, 26)
Clavispora (1, 1)	*Lipomyces* (2, 2)	*Stephanoascus* (1, 1)
Cryptococcus (28, 37)	*Lodderomyces* (1, 1)	*Sterigmatomyces* (2, 3)
Cystofilobasidium (3, 3)	*Malassezia* (2, 2)	*Taphrina* (6, 6)*
Debaryomyces (7, 9)	*Mastigobasidium* (1, 1)	*Tilletia* (1, 1)
Dekkera (1, 1)	*Mastigomyces* (1, 1)	*Tilletiaria* (1, 1)
Diaporthe (1, 1)	*Metschnikowia* (4, 4)	*Tilletiopsis* (5, 6)*
Dioszegia (2, 9)	*Microbotryium* (5, 7)	*Torulaspora* (1, 1)
Dipodascus (1, 1)	*Mrakia* (2, 4)	*Trichosporon* (12, 13)
Endomyces (1, 1)	*Myxozyma* (4, 4)	*Trimorphomyces* (1, 1)
Endophyllum (1, 1)	*Nadsonia* (1, 1)	*Tsuchiyaea* (1, 1)
Erythrobasidium (1, 1)	*Pichia* (1, 1)	*Udeniomyces* (3, 12)
Exobasidium (4, 5)	*Protomyces* (2, 2)	*Ustilago* (7, 7)*
Farysia (1, 1)	*Pseudozyma* (3, 8)*	*Wickerhamia* (1, 1)
Fellomyces (3, 3)	*Puccinia* (1, 1)	*Williopsis* (1, 1)
Filobasidiella (1, 50)	*Rhodosporidium* (4, 4)	*Xanthophyllomyces* (1, 4)
Filobasidium (3, 3)	*Rhodotorula* (37, 46)*	*Zygosaccharomyces* (1, 1)
Guilliermondella (1, 1)	*Saturnispora* (1, 1)	*Zygozyma* (1, 1)

Notes: The asterisks mark the genera that include resistant species. Figures in parentheses indicate the number of species and strains tested.

glucose in the hydrolysates was maximum, whereas cellobiose was absent. The hydrolysates sometimes contained mannose in trace amounts.

The antifungal activity of the purified preparation was proportional to its concentration (Fig. 3). The activity was fungicidal, as is evident from the fact that the 15-min incubation (pH 4.0; 20°C) of the preparation added to a suspension of *Microbotryum silenes-inflatae* VKM Y-2974 cells (7×10^6 cells/ml) to a concentration of 0.05 mg/ml diminished the content of viable cells by 93%. After the next 15 min of such incubation, only 0.5% of the cells remained viable. When stored at 4°C, the purified preparation remained active for several months.

DISCUSSION

In many cases, antagonistic interactions between yeasts are due to extracellular proteins (mycocins), whose synthesis is often determined by viruses and plasmids [11]. The antifungal activity of mycocins manifests itself at acidic pH values of the medium. According to the data of amino acid and sugar analyses, the hydrolysates of the low-molecular-weight antifungal agent of *Symp. paphiopedili* contained only sugars, whereas ^{1}H NMR spectroscopy showed the presence of functional groups typical of fatty acids (Fig. 2). Consequently, the antifungal agent of *Symp. paphiopedili* is likely to be a glycolipid. The ^{13}C NMR and mass spectroscopy of this substance showed the presence of cellobiose and 2,15,16-trihydroxypalmitic acid [12]. Analogous cellobiose lipids were earlier revealed in *U. maydis* [13] and *Cr. humicola.* These glycolipids may intercalate into the lipid matrix [6] and alter the permeability of membranes, because of which the cell loses vitally important compounds and dies [7].

The failure of our attempts to eliminate the antifungal phenotype of strain VKM Y-2817 suggests that the synthesis of its cellobiose lipid is controlled by chromosomal genes. Unlike mycocins, whose action is taxon-specific and limited to phylogenetically related organisms, the cellobiose lipid of strain VKM Y-2817 is active against a wide range of organisms. This distinguishes the antagonism of strain VKM Y-2817 from mycocinogeny. The antifungal agent of this strain is active against both ascomycetous and basidiomycetous

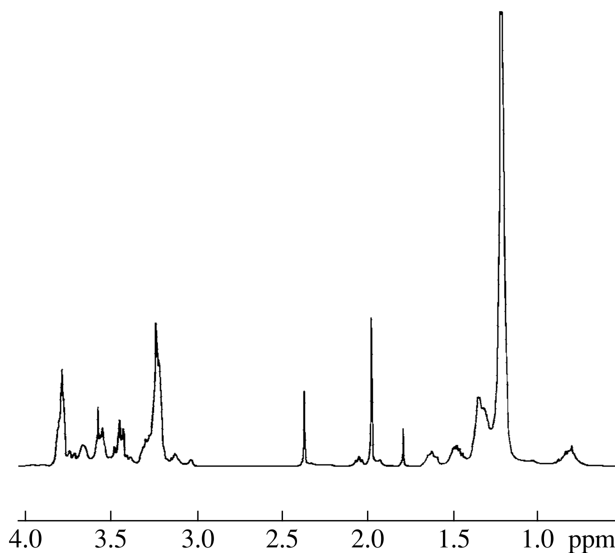

Fig. 2. The ^{1}H NMR spectrum of the antifungal preparation purified from *Symp. paphiopedili* VKM Y-2817.

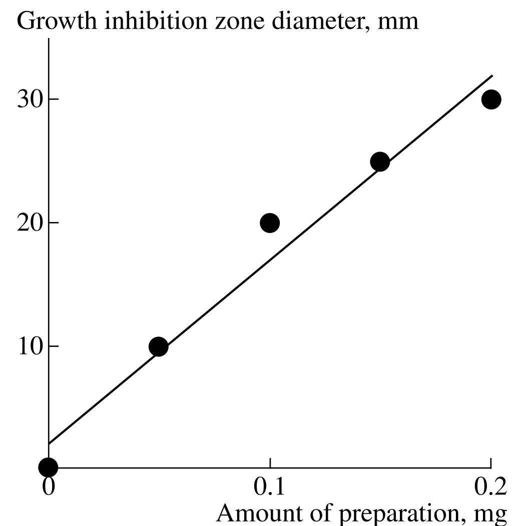

Fig. 3. The dependence of the diameter of the growth inhibition zone of *Cryptococcus terreus* VKM Y-2253 cells on the amount of the applied purified antifungal preparation of *Symp. paphiopedili* VKM Y-2817.

fungi (table), although the latter are more sensitive than the former. Indeed, the growth inhibition zones of ascomycetes are typically smaller than those of basidiomycetes. Furthermore, the relative number of resistant ascomycetous fungi is about one-third, whereas the relative number of resistant basidiomycetous fungi does not exceed 7%. This difference can be accounted for by the different lipid compositions of the membranes of ascomycetes and basidiomycetes [14, 15] since the cytoplasmic membranes of these fungi are primary targets for extracellular cellobiose lipids.

It should be noted that extracellular cellobiose lipids and their producers are considered to be promising fungal biocontrol agents [5]. Our earlier studies with a wide range of fungi [4, 16] showed that such agents may also be used in medicine and veterinary. In particular, the glycolipid of *Symp. paphiopedili* is active against many phytopathogens (*Diaporthe, Endophyllum, Exobasidium, Farysia, Gymnosporangium, Microbotryum, Protomyces, Puccinia, Sclerotinia* (Fig. 1), *Sorosporium, Sphacelotheca, Sporisorium, Taphrina, Tilletia*, and *Ustilago*), as well as against some causal agents of animal and human mycoses (*Candida*, *Clavispora, Filobasidiella, Malassezia*, and *Trichosporon*) (table).

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