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EXPERIMENTAL ARTICLES

Mycocinogeny in Smut Yeast-Like Fungi of the Genus *Pseudozyma*

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Abstract—The fungistatic agent secreted by *Pseudozyma prolifica* VKM Y-2835 shows activity against some representatives of the *Ustilaginales* under acidic conditions. This mycocin, with a molecular mass of no less than 15 kDa, is thermolabile and sensitive to proteolytic cleavage.

Key words: antagonism, mycocin, killer toxin, *Pseudozyma, Ustilaginales*.

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The genus *Pseudozyma* Bandoni, established in 1985 with the only species *P. prolifica* [1], was radically changed ten years later. Six yeast-like basidiomycete anamorphic species phylogenetically related to the *Ustilaginales*, which had been described previously as the members of the genera *Candida, Sporobolomyces, Sporothrix, Sterigmatomyces*, and *Trichosporon* were transferred to this genus [2]. At present, the genus *Pseudozyma* Bandoni emend. Boekhout includes over ten species [3], many of which have attracted attention of biotechnologists as promising producers of enzymes [4, 5], heterologous proteins [6], a biologically active glycolipid with the carbohydrate part containing a disaccharide of mannose and erythritol [7], and itaconic acid [8], as well as antagonists of phytopathogenic fungi [9].

The antifungal activity of *Pseudozyma* species is determined by secretion of cellobiose lipids with a fungicidal activity against a very broad spectrum of fungi [9]. At the same time, *P. tsukubaensis* has been shown to produce a killer toxin (mycocin) with a very narrow, taxonomically specific action spectrum: it is active only against some representatives of the orders *Microstromatales* and *Ustilaginales* [10]. Recently, we have uncovered similar activity in *P. prolifica*, the type species of this genus. The conditions of its manifestation, the nature of the antifungal factor, and the spectrum of sensitive species are characterized in this work.

MATERIALS AND METHODS

Strains. The strains used in this work were mainly obtained from the All-Russia Collection of Microorganisms (VKM, http://www.vkm.ru). The cultures of *Ustilago maydis* 27P1 and 75P6 were obtained from the collection of the Chair of Microbiology, University of Tel Aviv (Israel). The strains of *P. fusiformata* designated L1 and PTZ were isolated at our laboratory. All strains were maintained on malt agar (MA).

Sensitivity testing. Three-day cultures grown on MA at 20°C were used for the assay. Their sensitivity to the toxin secreted by *P. prolifica* VKM Y-2835 was tested by the method of "culture vs. culture" on glucose–peptone agar (GPA) with a citrate–phosphate buffer under incubation at 18°C [10].

Obtaining the toxin. A glucose–peptone medium (GPA, pH 4.0) without glycerol and agar was used to obtain the toxin. *P. prolifica* VKM Y-2835 was grown for four days in a shaker (150 rpm) or for 10 days without shaking. The cells were separated by centrifugation (3000 *g*, 10 min, 5°C), and the supernatant was filtered through GF/A glass filters (Sigma, United States).

Characteristics of the toxin. The effect of the toxin on the sensitive cells was determined by incubation with the obtained toxin-containing culture liquid; cell viability was determined by plating on MA. This culture liquid was also used to test resistance of the toxin to high temperatures and proteolytic cleavage by the agar well method. To determine the diffusion of the toxin through dialysis membranes permeable to substances of certain molecular masses (Spectrum, United States), strain VKM Y-2835 was grown on a GPA medium (pH 4.0) covered with these membranes. After a week's incubation (18°C), the membrane was removed along with the streak culture and the GPA surface was inoculated with the toxin-sensitive strain *P. fusiformata* PTZ-351.

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Table 1. Action spectra of the mycocins of *Pseudozyma prolifica* VKM Y-2835 and *Pseudozyma tsukubaensis* VKM Y-2190

Species, strain numbers	2835^T	2190^T*
Farysia thuemenii VKM Y-2686	w	–
Pseudozyma antarctica BKM Y-2604^T	–	–
P. aphidis VKM Y-2090^T	–	–
P. fusiformata VKM Y-2821^T, 2909	–	–
Ll-41, 71	w	w
VKM Y-2898, PTZ-356, Ll-16	+	w
PTZ-351	+	+
P. graminicola VKM Y-2938^T, Ll-46	–	–
P. prolifica VKM Y-2835^T	x	–
P. tsukubaensis VKM Y-2190^T	–	x
Rhodotorula acheniorum VKM Y-2213^T	–	–
R. bacarum VKM Y-2171^T	–	w
R. hinnulea VKM Y-2664, 2665^T	–	+
Sporisorium tranfissum VKM Y-2692	+	+
Tilletiopsis albescens VKM Y-2822^T, F-3165	–	+
T. flava VKM Y-2823	–	–
T. washingtonensis VKM F-2951	–	–
Ustilago cordae VKM F-2967	–	–
U. cynodontis VKM F-2968	–	–
U. filiformis VKM F-2970	–	–
U. hordei VKM F-2969	w	–
U. maydis VKM F-2971, 27P1	–	–
75P6	–	w
U. perennans VKM F-2972	+	w

Note: T, type strain; +, sensitive; w, weakly sensitive; –, insensitive.

* The data from [10].

Elimination of antifungal activity. For this purpose, 0.1 ml of cell suspension (10^4 cells/ml) of strain VKM Y-2835 was plated as a lawn on MA and incubated at 35°C. In two weeks, several tens of randomly selected colonies were tested for presence of activity.

Nucleic acid analysis. Methods of isolation and electrophoretic analysis of nucleic acids have been described previously [11].

RESULTS

The antifungal activity of *P. prolifica* VKM Y-2835 was revealed under cross testing of the cultures of *Pseudozyma* species. Judging from the width of growth inhibition zones, strain *P. fusiformata* PTZ-351 showed the highest sensitivity. Using this strain as a tester, it was shown that the antifungal activity of strain VKM Y-2835 was detected only at acidic pH values, 3.5 to 5.0. The growth inhibiting activity was very weak at pH 5.0 and was absent at higher values (5.5 and 6.0). The maximal zones of growth inhibition were observed at pH 4.0. The composition of the medium (GPA, MA) and buffer (citrate–phosphate, sodium succinate) had no significant effect on the antifungal activity. The growth inhibiting factor was present when strain VKM Y-2835 was grown on agarized or in liquid media, both with and without shaking.

This factor proved to be heat-sensitive: it was completely inactivated after 5 min heating at 100°C. The activity was also lost after treatment with pronases E and P (Sigma, United States). However, the toxin was resistant to pepsin and trypsin. It did not diffuse through the dialysis membrane, which was impermeable to compounds with molecular masses of 15 kDa and more.

The toxin under study exhibited fungistatic activity. Repeated inoculations on MA of the cell suspension (2×10^3 cells/ml) incubated for two days in the toxin-containing culture liquid of *P. prolifica* VKM Y-2835 showed no decrease of the quantity of viable cells of *P. fusiformata* PTZ-351.

The toxin secreted by *P. prolifica* had a very narrow and taxonomically specific action spectrum. It was active only against some representatives of the order *Ustilaginales*, class *Ustilaginomycetes* (Table 1). All the other 266 strains tested, which belong to 205 species of 67 genera of ascomycetous and basidiomycetous fungi, were insensitive to it (Table 2).

The antifungal activity of strain VKM Y-2835 was not eliminated after incubation at a higher temperature close to the maximal one for its growth on MA (36°C). Electrophoresis in agarose gel of the total preparation of nucleic acids of this strain also showed no presence of plasmid RNA or DNA.

DISCUSSION

Thermolability, sensitivity to proteases, and substantial molecular mass of the antifungal agent produced by *P. prolifica* VKM Y-2835 indicate its proteinaceous nature. This fact, as well as the specific spectrum of organisms sensitive to it (Tables 1, 2), makes it possible to assign the toxin secreted by strain VKM Y-2835 to mycocins, the fundamental characteristics of which is activity against taxonomically related organisms [9]. These data are in good agreement with the results of rDNA sequencing demonstrating the phylogenetic affiliation of *Pseudozyma* species with *Ustilaginaceae* [12]. At the same time, these data show that they are rather far from each other phylogenetically and are distributed among several teleomorphic genera: *Cintractia, Moesziomyces, Sporisorium*, and *Ustilago* [3]; i.e., the genus *Pseudozyma* in its current understanding is taxonomically heterogeneous. Insensitivity of its species (except for *P. fusiformata*) to the mycocins of *P. prolifica* and *P. tsukubaensis* confirms this conclusion (Table 1). The reasons for strain vari-

Table 2. The fungi insensitive to the mycocin of *Pseudozyma prolifica* VKM Y-2835 (the numbers of tested species and strains, respectively, are given in brackets)

Arthroascus (2, 2)	*Nadsonia* (2, 2)
Bensingtonia (9, 9)	*Nematospora* (1, 1)
Bulleromyces (1, 2)	***Neovossia* (2, 2)**
Candida (8, 9)	*Pachysolen* (1, 1)
Citeromyces (1, 2)	*Phomopsis* (1, 1)
Cryptococcus (20, 33)	*Pichia* (2, 2)
Curvibasidium (1, 1)	*Protomyces* (2, 2)
Cystofilobasididum (3, 3)	*Puccinia* (3, 3)
Debaryomyces (12, 13)	*Rhodosporidium* (8, 14)
Dioszegia (2, 2)	*Rhodotorula* (16, 44)
***Entyloma* (1, 1)**	*Saccharomyces* (12, 12)
Erythrobasidium (1, 1)	*Sakaguchia* (1, 2)
***Exobasididum* (8, 9)**	*Schizosaccharomyces* (2, 4)
Fellomyces (1, 1)	*Sclerotinia* (1, 1)
Fibulobasidium (1, 1)	*Sirobasidium* (1, 1)
Filobasidiella (1, 1)	*Sphacelotheca* (1, 1)
Filobasididum (1, 1)	*Sporidiobolus* (5, 6)
Geotrichum (1, 1)	*Sporobolomyces* (4, 4)
Guilliermondella (1, 1)	*Sterigmatomyces* (1, 1)
Hanseniaspora (2, 2)	*Sterigmatosporidium* (1.1)
Holtermannia (1, 1)	***Sympodiomycopsis* (1, 1)**
Itersonilia (1, 1)	*Taphrina* (8, 8)
Kockovaella (1, 1)	***Tilletia* (1, 1)**
Kondoa (1, 1)	*Torulaspora* (3, 3)
Kurtzmanomyces (1, 1)	*Tremella* (2, 2)
Leucosporidium (2, 2)	*Trichosporon* (9, 9)
Lipomyces (2, 2)	*Wickerhamia* (1, 1)
Lodderomyces (1, 1)	*Wickerhamiella* (1, 1)
Mastigobasidium (1, 1)	*Williopsis* (2, 2)
Mastigomyces (1, 1)	*Xanthophyllomyces* (1, 2)
Metschnikowia (7, 10)	*Zygoascus* (1, 1)
Microbotryum (6, 7)	*Zygosaccharomyces* (1, 2)
Microstroma (1, 1)	*Zygowilliopsis* (1, 1)
Mrakia (2, 2)	

Note: smut fungi are in bold.

ability of the sensitivity of *P. fusiformata* are still unclear.

The synthesis of mycocin in *P. prolifica* is apparently determined by chromosomal genes, because incubation at a high temperature, which facilitates elimination of the cytoplasmic genetic elements, did not result in the loss of activity; the analysis of total nucleic acids did not show the presence of such elements.

At present, mycocin formation has been reported in two species of the genus *Pseudozyma*: *P. antarctica* [13] and *P. tsukubaensis* [10]. In contrast to the toxin characterized in this work, the mycocin of the latter species shows fungicidal activity, is resistant to proteolysis, and has a slightly different action spectrum (Table 1). As regards *Ps. antarctica*, the antifungal agent of an unknown chemical nature produced by this species is likely not a mycocin, because it is active against quite different and taxonomically distant organisms (*Candida, Cryptococcus, Filobasidiella, Kluyveromyces, Saccharomyces,* and *Yarrowia*). Apparently, it belongs to a category of extracellular glycolipids possessing antifungal activity [14]; the synthesis of such compounds is widespread among *Ustilaginales* [9]. It is not improbable that the glycolipid-secreting *Pseudozyma* species can also form mycocins. However, it is difficult to detect them in such cases, because glycolipids have much broader action spectra, which complicate the detection of mycocinogenic activity.

Among the representatives of *Ustilaginomycetes*, mycocinogeny has also been described in species of the genus *Tilletiopsis* [15, 16], but has been most thoroughly studied in *U. maydis* [17]. In contrast to anamorphic organisms of the genera *Pseudozyma* and *Tilletiopsis*, the synthesis of all three types of mycocins in the latter species is determined by extrachromosomal genetic elements, namely dsRNA-carrying viruses. It is notable that mycocinogeny in teleomorphic yeasts, both ascomycetous and basidiomycetous, is in most cases determined by the presence of viruses or plasmid DNA in the cells, whereas the synthesis of mycocins in anamorphic yeasts seems to be determined by nuclear genes.

Pseudozyma species are usually described as saprotrophic epiphytes. Very likely, secretion of antifungal agents not only increases their competitiveness in the phylloplane microbiota but also is an important natural element of plant protection from pathogenic fungi [9, 18].

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