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EXPERIMENTAL ARTICLES

Specificity of Yeast Sensitivity to the Mycocin of *Tilletiopsis flava* VKM Y-2823

V. I. Golubev and L. G. Churkina

All-Russia Collection of Microorganisms, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia

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Abstract—The mycocinogenous strain *Tilletiopsis flava* VKM Y-2823 was found to possess fungicidal activity at pH 3.5–4.5, which was retained after curing the strain by eliminating the extrachromosomal genetic elements. The mycocin produced by the strain had a molecular mass of more than 10 kDa and was readily inactivated by heating and treatment with protease K. This mycocin was found to be active against species of the anamorphic genus *Tilletiopsis.* The overwhelming majority of other representatives of the order *Tilletiales*, as well as ascomycetous and basidiomycetous (including ballistosporous) yeasts of the orders *Sporidiales* and *Tremellales*, were resistant to it.

Key words: *Tilletiopsis*, killer toxin, taxonomy, identification, antagonism.

Ballistospore-forming yeastlike fungi are typical of the phyllosphere mycobiota. Contrary to the popular view that this mycobiota includes related microorganisms, phylogenetic analysis revealed that ballistospore-forming fungi belong to different basidiomycetous orders (*Sporidiales*, *Tilletiales*, and *Tremellales*) and classes (*Hymenomycetes*, *Urediniomycetes*, and *Ustilaginomycetes*) [1–5]. These findings have not yet been employed in the practical taxonomy of ballistospore-forming basidiomycetes, which is still based on conventional, often variable, characteristics (pigmentation, the form and size of ballistospores, the type of mycelium, and the range of utilizable carbon and nitrogen sources [6]). Moreover, basidiomycetes often lose the ability to form ballistoconidia, which makes it very difficult to distinguish ballistospore-forming yeastlike fungi from phenotypically similar nonballistospore-forming ones. In such cases, taxonomically and phylogenetically related mycocinogenous strains can be differentiated by the so-called mycocinotyping [6], whose specificity, however, depends on the diversity and amounts of mycocins produced. Until now, only two such fungi, *Ustilago maydis* and *Tilletiopsis albescens*, have been reported to produce killer toxins [7, 8].

Earlier, the type strain of ballistospore-forming smut fungi, *Tilletiopsis flava* (Tubaki) Boekhout VKM Y-2823, was found to produce mycocin. This paper presents the results of the investigation of some properties of this mycocin.

MATERIALS AND METHODS

The cultures used in this study (predominantly type strains) were obtained from the All-Russia Collection of Microorganisms (VKM) and the Japanese Collection of Microorganisms (JCM).

The mycocin susceptibility of strains was tested at 20°C by the culture-to-culture method using glucose–peptone agar (GPA) [8] prepared with a sodium–succinate buffer (pH 4.0). To obtain a mycocin-containing culture liquid, strain VKM Y-2823 was grown at 20°C for one month in nonagitated flasks in a glucose–peptone liquid medium, after which the fungal biomass was removed by centrifugation. The supernatant was filtered through a GF/A membrane (Whatman, United Kingdom) and used for the estimation of the resistance of mycocin to elevated temperatures and proteolysis by the method of agar wells. The number of the viable propagules of test cultures after incubation with the mycocin-containing culture liquid of strain VKM Y-2823 was determined by plating the appropriate dilutions of incubation mixtures in triplicates on wort agar. The results were expressed as colony-forming units (CFU).

To determine the molecular mass of mycocin, strain VKM Y-2823 was grown on membranes with different pore sizes (Spectrum, United States) laid on the surface of the GPA. The membranes with grown colonies were removed, and a mycocin-sensitive test culture was grown on the surface of the GPA to detect whether or not it would be inhibited by the mycocin passed through the membranes.

Extrachromosomal genetic elements were eliminated from strain VKM Y-2823 by incubating it at 30°C (this temperature is close to the highest temperature at which the strain can grow) or in a medium with 100 μg/ml cycloheximide.

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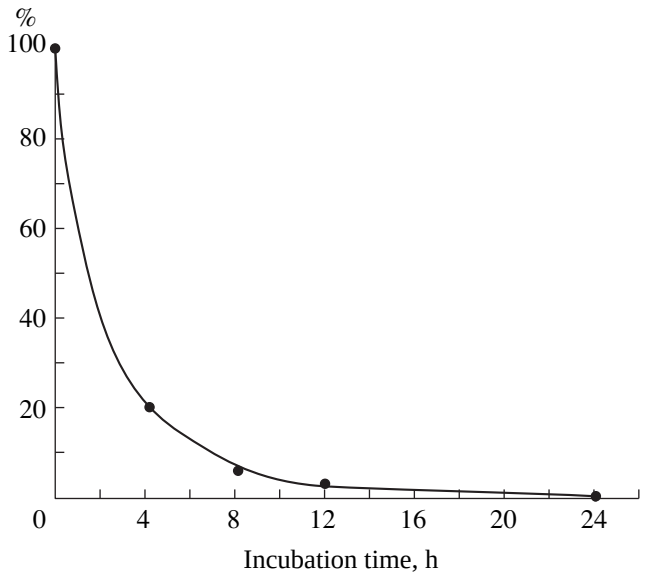

Death curve of the *Tilletiopsis flava* VKM Y-2822 cells incubated at 20°C in the mycocin-containing culture liquid (pH 4.0) of *T. flava* VKM Y-2823. The initial concentration of viable *T. flava* VKM Y-2822 cells was 1.9×10^4 CFU/ml.

RESULTS AND DISCUSSION

The killer activity of strain VKM Y-2823 was tested by incubating it with the type strains of the *Tilletiopsis* Derx species. The activity was high at pH values between 3.5 and 4.5, and was not detected at pH 5.0 or higher. The extracellular toxin was found to be lethal to sensitive cultures (see figure).

The toxin was stable at low temperatures and the killer activity of the toxin-containing culture liquid did not considerably change during storage at 5°C for one year. At 100°C, however, the toxin was completely inactivated within 1 min. The toxin was readily inactivated by proteinase K (Sigma, United States) and did not penetrate through membranes with a nominal molecular weight cutoff of 6–8 and 10 kDa. These data suggest that the *T. flava* VKM Y-2823 toxin is a protein with a molecular mass of more than 10 kDa. Such protein toxins of yeasts and yeastlike fungi with fungicidal activity at acidic pH values are known as killer toxins [6]. The relatively high molecular mass and activity against fungi (the data are presented below) allow the *T. flava* VKM Y-2823 toxin to be referred to as mycocin [9].

The testing of 356 strains of 282 species belonging to 79 genera of yeasts and yeastlike fungi for their susceptibility to the *T. flava* VKM Y-2823 mycocin showed that its action is taxonomically specific: it was inactive against all of the investigated ascomycetes (including archiascomycetes), hymenomycetes, and urediniomycetes, including the ballistospore-forming species of the genera *Bensingtonia*, *Bullera*, *Bulleromyces*, *Entyloma*, *Itersonilia*, *Kockovaella*, *Mastigobasidium*, *Sporidiobolus*, *Sporobolomyces*, *Tilletia*, *Tilletiaria*, and *Udeniomyces* (Table 1), and active against ustilaginomycetes of the subclass *Exobasidiomycetidae* (mainly against the species of the anamorphic genus *Tilletiopsis*) (Table 2). Teleomorphic and anamorphic smut fungi of the genera *Farysia, Malassezia, Neovossia, Pseudozyma, Sorosporium, Sphacelotheca, Sporisorium, Sympodiomycopsis*, and *Ustilago*, as well as of the teleomorphic genera *Entyloma, Tilletia*, and *Tilletiaria* [2, 4], which are phylogenetically very close to *Tilletiopsis*, were found to be mycocin-resistant (Table 1). Among ustilaginomycetes, only *Exobasidium vaccinii*, *Rhodotorula hinnulea*, and *R. phylloplana* were slightly sensitive to the *T. flava* mycocin. These species exhibited growth inhibition zones of about 1 mm in diameter that disappeared during prolonged incubation (Table 2). This finding agrees with the data of rRNA sequence analysis, according to which the overwhelming majority of the *Rhodotorula* species belong to the order *Sporidiales* [1], while *R. hinnulea* and *R. phylloplana* are related to smut fungi [8].

The characteristic feature of mycocins is their activity against the microorganisms related to the mycocin producer, although the degree of relatedness of mycocin-sensitive microorganisms may be different [6]. As for the *T. flava* mycocin, it is active against all of the investigated species of the genus *Tilletiopsis*, and various strains of particular species exhibit a similar sensitivity to the mycocin (Table 2). The mycocin resistance of *T. palescens* not only fails to contradict this inference but even confirms it, since the assignment of this species to the genus *Tilletiopsis* is questionable. Indeed, the G+C content of the *T. palescens* DNA is 42–45 mol %, compared to 55–70 mol %, which is typical of other members of this genus [10]. Furthermore, the results of the large subunit rRNA sequence show that this species is distant from other *Tilletiopsis* species [2]. As for *T. minor*, this species is closely related to *T. flava*, so that the latter was even proposed to be considered as *T. minor* var. *flava*. The tolerance of *T. minor*, as well as of the producer *T. flava* VKM Y-2823, to mycocin can be explained by their immunity to this protein.

All 350 clones obtained by eliminating the extrachromosomal genetic elements from *T. flava* VKM Y-2823 retained the ability to produce mycocin. This finding suggests that genes responsible for the synthesis of mycocin in *T. flava* are located on chromosomes.

Reportedly, *Tilletiopsis* species are antagonistic to some mildews [11, 12]. The mechanism of this phenomenon remains unknown. Indeed, there is evidence that these species lack cellular structures typical of mycopathogens; they also do not produce substances toxic to mildews. The production of mycocins cannot explain the antiphytopathogenic activity of the *Tilletiopsis* species either, since the mycocins of the two known mycocin producers of the genus *Tilletiopsis*, *T. albescens* [8] and *T. flava*, are inactive against ascomycetes. The production of mycocins by the *Tilletiopsis* species most probably augments their competitive-

Table 1. Fungal genera whose species are tolerant to the mycocin of *T. flava* VKM Y-2823

Agaricostilbum (1, 1)	*Microbotyum* (7, 8)
Ambrosiozyma (1, 1)	*Mrakia* (2, 3)
Arthroascus (1, 1)	*Myxozyma* (4, 4)
Atractogloea (1, 1)	*Nadsonia* (1, 1)
Arxula (1, 1)	*Neovossia* (2, 2)
Bensingtonia (6, 6)	*Pichia* (1, 1)
Bullera (14, 33)	*Platygloea* (1, 1)
Bulleromyces (1, 2)	*Protomyces* (2, 2)
Calocera (1, 1)	*Pseudozyma* (6, 6)
Candida (3, 3)	*Puccinia* (3, 3)
Citeromyces (1, 1)	*Rhodosporidium* (3, 3)
Clavispora (1, 1)	*Rhodotorula** (34, 41)
Cryptococcus (38, 40)	*Saccharomyces* (1, 1)
Cystofilobasidium (4, 4)	*Saturnospora* (1, 1)
Dacrymyces (1, 1)	*Schizosaccharomyces* (3, 16)
Debaryomyces (7, 9)	*Septobasidium* (1, 1)
Dipodascus (1, 1)	*Sorosporium* (1, 1)
Endophyllum (1, 1)	*Sphacelotheca* (1, 1)
Entyloma (1, 1)	***Sporidiobolus*** (4, 4)
Erythrobasidium (1, 1)	*Sporisorium* (2, 3)
*Exobasidium** (7, 7)	***Sporobolomyces*** (24, 27)
Farysia (1, 1)	*Stephanoascus* (1, 1)
Fellomyces (3, 3)	*Sterigmatomyces* (2, 2)
Filobasidium (2, 2)	*Sympodiomycopsis* (1, 1)
Guepiniopsis (1, 1)	*Taphrina* (11, 11)
Guilliermondia (1, 1)	*Tausonia* (1, 1)
Gymnosporangium (1, 1)	***Tilletia*** (1, 1)
Hanseniaspora (1, 1)	***Tilletiaria*** (1, 1)
Holtermannia (1, 1)	*Torulaspora* (1, 1)
Issatchenkia (1, 1)	*Trichosporon* (11, 13)
Itersonilia (1, 2)	*Trimorphomyces* (1, 1)
Kockovaella (1, 1)	*Tsuchiyaea* (1, 1)
Kurtzmanomyces (1, 1)	***Udeniomyces*** (3, 12)
Leucosporidium (2, 2)	*Ustilago* (12, 17)
Lipomyces (3, 3)	*Wickerhamia* (1, 1)
Lodderomyces (1, 1)	*Williopsis* (1, 1)
Malassezia (2, 2)	*Xanthophyllomyces* (1, 4)
Mastigobasidium (1, 1)	*Zygosaccharomyces* (1, 1)
Mastigomyces (1, 1)	*Zygozyma* (1, 1)

Note: Ballistospore-forming organisms are shown in bold. Numbers in parentheses indicate the number of species and strains investigated. The asterisk marks the *Rhodotorula* genus, whose particular species are slightly sensitive to the mycocin (see Table 2).

Table 2. Some fungal strains that are sensitive, slightly sensitive, and insensitive to the mycocin of *T. flava* VKM Y-2823

Exobasidium vaccinii VKM F-3317	±
Rhodotorula hinnulea VKM Y-2665T	±
R. phylloplana VKM Y-2664T	±
Tilletiopsis albescens VKM Y-2822T	+
T. albescens VKM F-3165	+
T. fulvescens JCM 5187T	+
T. minor JCM 5185	–
T. minor JCM 8361T	–
T. palescens JCM 5230T	–
T. washingtonensis JCM 5184 (*T. cremea*, T)	+
T. washingtonensis JCM 8362T	+
T. washingtonensis VKM F-2951	+

Note: VKM, All-Russia Collection of Microorganisms; JCM, Japan Collection of Microorganisms; T, type strain. "+", "±", and "–" stand for "sensitive", "slightly sensitive", and "insensitive", respectively.

ness with related species occupying close ecological niches.

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