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EXPERIMENTAL ARTICLES

Taxonomic Specificity of the Sensitivity to the *Wickerhamomyces bovis* Fungistatic Mycocin

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Abstract—*Wickerhamomyces bovis* type strain was found to secrete a mycocin with a fungistatic effect at pH from 3.5 to 6.0. The peak of its activity occurred at pH 5.0 in the presence of 3% NaCl. Yeast species sensitive to this mycocin were located within the family *Wickerhamomycetaceae* and belonged to phylogenetically related genera *Ambrosiozyma, Nakazawaea, Ogataea,* and *Peterozyma*.

Keywords: yeasts, mycocinogeny, killer toxin, taxonomy

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Formation of extracellular antifungal (glyco)peptides (mycocins) is widespread among yeasts (Golubev, 2012) and was revealed, in particular, in some species of *Wickerhamomyces* Kurtzman et al.: *W. anomalus* (Hansen) Kurtzman et al., *W. canadensis* (Wickerham) Kurtzman et al., *W. ciferrii* (Lodder) Kurtzman et al., *W. silvicola* (Wickerham) Kurtzman et al., *W. subpelliculosa* (Kurtzman) Kurtzman et al., (Young and Yagiu, 1978; Polonelli et al., 1983; Golubev, 2015, 2015b, 2015c). According to the available data, the mycocins (killer toxins) of these species with molecular mass ranges from several dozens to hundreds kDa are encoded by chromosomal genes and exhibit fungicidal activity at low ambient pH values (4–6) against the organisms, which are phylogenetically related to mycocinogenic strains. The latter, especially those belonging to the species *W. anomalus,* have attracted close attention from a practical point of view: for typing candidiasis pathogens and suppressing the fungal spoilage of feeds and foodstuffs (Passoth et al., 2011).

Recently, it was found that, among *Wickerhamomyces* spp., *W. canadensis* type strain was sensitive to most antifungal agents produced by *W. anomalus* cultures (Golubev, 2015b). When it was used as a tester, the previously unknown antibiotic activity was revealed in the *W. bovis* type strain (van Uden et do Carmo-Sousa) Kurtzman et al.

The goal of the present work was to study conditions for the expression of antibiotic activity, the spectrum of action, and other characteristics of the antifungal agent secreted by *Wickerhamomyces bovis* type strain.

MATERIALS AND METHODS

Since all the strains used in this study are maintained in the All-Russian Collection of Microorganisms (http://www.vkm.ru/), the abbreviation (BKM Y-) before their numbers is therefore omitted.

Sensitivity to the mycocin was tested on glucose–peptone agar (g/L): glucose, 5; peptone, 2.5; yeast extract, 2; agar, 20 with citrate–phosphate buffer. The suspension (0.05 mL, approximately 10^5 cells/mL) of the 24–72-h cultures grown on malt agar was applied on its surface and thoroughly spread with a spatula, and then streaked with an abundant inoculum of *W. bovis* 1106 48–72-h culture (Table 1). Inoculated dishes were incubated at room temperature until lane growth became visible. When a growth inhibition zone several millimeters wide was formed round the streak, the studied culture was recorded as sensitive; if it did not exceed 1–2 mm, the culture was considered weakly sensitive; and if growth inhibition was absent, it was insensitive.

For production of the antifungal agent, strain 1106 was grown under static conditions in the same liquid glucose–peptone medium with glycerol (50 mL/L) for about one month. The cells were separated by centrifugation (5000 *g*, 10 min), and the supernatant fluid was filtered through GF/A glass filters (Sigma, United States). The culture liquid thus obtained was used for testing the agent's resistance to elevated temperature, for proteolysis using the agar well method, as well as for assessing its action on the viability of sensitive cells, which was determined in the process of incubation by plating on malt agar.

Table 1. Genera of the yeasts whose species are insensitive to the mycocin of *Wickerhamomyces bovis* (the number of the species and strains studied)

Genus	Number of tested species and strains	Genus	Number of tested species and strains	Genus	Number of tested species and strains
Babjeviella	(1, 1)	*Komagataella*	(2, 2)	*Saturnispora*	(1, 1)
Bensingtonia	(1, 1)	*Kondoa*	(1, 1)	*Scheffersomyces*	(1, 1)
Bulleromyces	(1, 1)	*Kregervanrija*	(1, 1)*	*Schizosaccharomyces*	(1, 2)
Citeromyces	(1, 1)	*Kuraishia*	(2, 4)	*Schwanniomyces*	(2, 2)
Clavispora	(1, 1)	*Kwoniella*	(1, 1)	*Sirobasidium*	(1, 1)
Cuniculitrema	(1, 1)	*Lachancea*	(1, 1)	*Sphacelotheca*	(1, 1)
Cystofilobasidium	(1, 1)	*Leucosporidium*	(2, 2)	*Sporidiobolus*	(1, 1)
Debaryomyces	(2, 2)	*Lipomyces*	(1, 1)	*Sporopachydermia*	(1, 1)
Dekkera	(2, 2)	*Metschnikowia*	(2, 2)	*Tausonia*	(1, 1)
Eremothecium	(1, 1)	*Meyerozyma*	(1, 2)	*Tetrapisispora*	(1, 1)
Farysia	(1, 1)	*Microstroma*	(1, 1)	*Tilletiopsis*	(1, 1)
Farysizyma	(1, 1)	*Millerozyma*	(1, 1)	*Torulaspora*	(1, 1)
Fellomyces	(1, 1)	*Nadsonia*	(1, 2)	*Tremella*	(1, 1)
Fibulobasidium	(1, 1)	*Naumovia*	(1, 1)	*Trichosporon*	(1, 1)
Filobasidiella	(1, 1)	*Naumovozyma*	(1, 1)	*Trimorphomyces*	(1, 1)
Filobasidium	(1, 1)	*Pachysolen*	(1, 1)	*Udeniomyces*	(1, 1)
Hannaella	(1, 1)	*Papiliotrema*	(1, 1)	*Vanderwaltozyma*	(1, 1)
Hanseniaspora	(5, 6)*	*Pichia*	(1, 1)	*Wickerhamiella*	(1, 1)
Holtermannia	(1, 1)	*Priceomyces*	(1, 1)	*Xanthophyllomyces*	(1, 1)
Hyphopichia	(1, 1)	*Rhodosporidium*	(1, 1)	*Yamadazyma*	(1, 1)
Kazachstania	(3, 3)	*Saccharomyces*	(1, 1)	*Yarrowia*	(1, 1)
Kluveromyces	(2, 2)	*Saccharomycodes*	(1, 1)	*Zygoascus*	(1, 1)
Kockovaella	(1, 1)	*Saccharomycopsis*	(2, 2)	*Zygosaccharomyces*	(1, 1)
Kodamaea	(1, 1)	*Sakaguchia*	(1, 1)	*Zygotorulaspora*	(2, 2)

* *H. valbyensis* (1) and *K. fluxuum* (1) are weakly sensitive. The strains are not included in the number of those indicated in the table.

For a rough estimate of the agent's molecular mass, strain 1106 was grown on the above-mentioned agar medium covered with a dialysis membrane (Spectrum, United States). After seven days of incubation, the membrane with the culture grown on it was removed, and the medium was inoculated with a lane of the sensitive strain *W. alni* 2509.

In order to eliminate antifungal activity, malt agar was inoculated with strain 1106 suspension (0.1 mL, 10^4 cells/mL) and incubated at the maximal growth temperature. Other dishes were subjected to UV irradiation (254 nm, ~200 J/m^2) for 2–6 min.

RESULTS AND DISCUSSION

Antifungal activity of *W. bovis* 1106 was revealed within the range of pH values from 3.5 to 6.0 (citrate–phosphate buffer). At pH 6.5 and higher it was not observed. The broadest growth inhibition zones of sensitive cultures were formed at pH 5.0. Their width increased when NaCl (3%) was added to the medium. The same effect, though slightly less pronounced, was observed when 50 mL/L glycerol was introduced into the medium. While the inhibition zones were even wider at a higher concentration of these compounds, growth of the culture slowed down. The glucose–peptone agar with 3% NaCl (pH 5.0) was therefore used for determination of the yeast species sensitivity to the agent produced by *W. bovis*. In the presence of methylene blue (0.03 g/L) in this medium, no blue rim indicating the penetration of the dye into dead cells was observed along the edge of the growth inhibition zone on the lawn of sensitive strains. Its absence indicated that the toxin possessed a fungistatic but not a fungicidal effect. Platings from the zone of complete growth inhibition after two weeks of incubation also demonstrated the presence of viable cells in it. During incubation in the toxin-containing *W. bovis* culture liquid, the number of viable cells of the sensitive strain did not

Table 2. Spectrum of action of *Wickerhamomyces bovis* mycocin among representatives of the family *Wickerhamomycetaceae*

Species	Reaction	Species	Reaction
Barnettozyma californica 838^T	w	*Cyb. subsufficiens* 2220^T	–
B. salictaria 1288	–	*Cyb. veronae* 2163	–
B. pratensis 2136^T	–	*Starmera amethionina* 2614^T	+
Candida berthetii 1430^T	–	*S. dryadoides* 2148	–
C. dendrica 2278	w	*S. pachycereana* 2616T, 2617	w
C. maritima 2593^T	–	*S. quercuum* 313, 1287^T	–
C. melinii 50T, 2357, 2358	+	*Wickerhamomyces alni* 2509T, 2510	+
C. odintsovae 2024, 2025^T, 2026, 2027	+	*W. anomalus* 157, 1086T, 1087, 1905, 2353, 2354, 2511*	–
C. silvicultrix 2189^T	–	*W. bisporus* 1065^T	+
C. solani 69T, 2334, 2335	–	*W. canadensis* 1395^T, 1399	+
		1400	w
C. quercuum 2157T, 2739	–	*W. chambardii* 276	w
C. vartiovaarae 2114, 2115, 2444, 2445, 2625^T	–	*W. ciferrii* 169^T	–
Cyberlindnera americana 1409^T	–	*W. lynferdii* 2205^T	+
Cyb. bimundalis 1407^T	+	*W. mucosus* 2086^T	–
Cyb. fabianii 1450^T	+	*W. pijperi* 310^T	–
Cyb. jadinii 33, 74, 768	–	*W. rabaulensis* 2197^T	+
Cyb. mrakii 173^T	–	*W. silvicola* 178^T, 1224, 2191	–
Cyb. petersonii 1410	w	*W. strasburgensis* 278, 2746	+
Cyb. rhodanensis 277	+	*W. subpelliculosa* 180^T, 1085, 1088-1091	+
Cyb. sargentensis 2087^T	–	*W. sydowiorum* 2192	+
Cyb. saturnus 2551^T	–	*W. wingei* 1398^T	–
Cyb. suaveolens 2990^T	–		

Designations: "+" indicates sensitive; "w", weakly sensitive; "–", insensitive; T, the type strain.
* The remaining 42 strains are usually weakly sensitive.

decrease, and even increased slightly after two days, probably due to the presence of residual nutrients.

The antifungal agent studied was completely inactivated after 5-min heating at 100°C, and its activity decreased when it was treated with pronase E (Serva, Germany). It did not penetrate through the dialysis membrane (Spectrum, United States), which does not allow the substances with a molecular mass of 8 kDa and higher to pass through. These data indicate the proteinaceous nature of the toxin secreted by *W. bovis*.

The antifungal activity of strain 1106 was not lost after treatments that eliminate extrachromosomal genetic elements. The study of 86 randomly selected clones obtained after UV irradiation and cultivated at the maximal growth temperature (37°C) showed that they all retained activity, i.e., the toxin synthesis in *W. bovis* is likely to be encoded by the chromosomal genes.

For their sensitivity to the toxin, 176 species from 82 genera (about 300 strains) were tested, and the results obtained give evidence of a narrow and taxon-specific spectrum of action of the agent produced. All the basidiomycetous yeasts tested, namely representatives of the classes *Agaricostilbomycetes*, *Cystobasidiomycetes*, *Exobasidiomycetes*, *Microbotryomycetes*, *Tremellomycetes,* and *Ustilaginomycetes*, were insensitive to the toxin. The predominant majority of ascomycetous yeasts in the classes *Saccharomycetes* and *Schizosaccharomycetes* of the families *Debaryomycetaceae*, *Lipomycetaceae*, *Metschnikowiaceae*, *Phaffomycetaceae*, *Pichiaceae*, *Saccharomycetaceae*, *Saccharomycodaceae*, *Saccharomycopsidaceae*, *Schizosaccharomycetaceae,* and *Trichomonascaceae* were also insensitive (Table 1). Almost all species sensitive to *W. bovis* toxin belonged to the family *Wickerhamomycetaceae* (Table 2), as well as to the genera *Ambrosiozyma* van der Walt, *Nakazawaea* Yamada et al., *Oga-*

Table 3. Spectrum of action of *Wickerhamomyces bovis* mycocin among the yeasts of unclear phylogenetic position

Species	Reaction	Species	Reaction
Ambrosiozyma angophorae 2218^T	–	*N. wickerhamii* 1489^T, 2706, 2870	–
A. llanquihuensis 2646^T	+	*Ogataea angusta* 1397^T	w
A. monospora 2383, 2202^T	–	*O. cecidiorum* 2982^T	–
A. philentoma 2067^T	–	*O. finlandica* 1404^T	–
A. platypodis 2082^T	+	*O. glucozyma* 2079^T	–
Candida boidinii 34, 1514, 1589, 2060, 2356, 2425, 2591^T	–	*O. henricii* 2080	–
2436, 2439	w		
C. etchelsii 1423^T, 1473, 1498, 2054	–	*O. methanolica* 2621^T	–
C. nemodendra 2174^T	–	*O. minuta* 172^T, 2085	–
C. nitratophila 1300^T	–	*O. naganishi* 2161^T	–
C. pini 2175^T	–	*O. nonfermentans* 2081^T, 2517	–
		2516	w
C. succiphila 2584^T	–	*O. parapolymorpha* 2518^T	+
C. maris 2107^T	w	*O. philodendra* 2168	w
C. sorboxylosa 2076^T	–	*O. pini* 886^T, 2495	+
		900, 2499	w
		899, 2496–2498, 2500–2503	–
Nakazawaea anatomiae 1522^T, 2709	–	*O. polymorpha* 2924, 2559T, 2988, 2989	w
N. ernobii 725^T	–	*O. trehalophila* 1289^T	–
N. holstii 1282, 1401, 1402	–	*O. wickerhamii* 2629	+
2005, 2558^T	w		
N. peltata 1482^T	w	*Peterozyma toletana* 2151^T	+

See Table 2 for designations.

taea Yamada et al., and *Peterozyma* Kurtzman et Robnett, the phylogenetic position of which and their related anamorphs remains unclear at present (Table 3). *H. valbyensis* Klöcker and *K. fluxuum* (Phaff et Knapp) Kurtzman were the only exceptions (Table 1), and *Lodderomyces elongisporus* (Recca et Mrak) van der Walt strains, including the type strain, were also sensitive. The candidiasis-causing pathogens *Candida albicans* (Robin) Berkhout, *C. glabrata* (Anderson) Meyer et Yarrow, *C. lodderae* (Phaff et al.) Meyer et Ahearn, *C. maltosa* Komagata et al., *C. parapsilosis* (Ashford) Langeron et Talice, *C. tropicalis* (Castellani) Berkhout, and *C. viswanathii* Sandhu et Randhawa appeared to be resistant.

The characteristics of the antifungal agent secreted by *W. bovis,* such as its activity at acidic ambient pH values, its proteinaceous nature, and, above all, the taxonomic specificity of the sensitivity make it possible to identify it as a mycocin (Golubev, 2012). In contrast to the predominant majority of known mycocins, which are fungicides, *W. bovis* mycocin exerts a fungistatic effect, similarly to the mycocin produced by *Cystobasidium pallidum* (Lodder) Yurkov et al. (=*Rhodotorula pallida* Lodder). The latter blocks bud formation in the sensitive cultures at the early stages of their formation (Golubev, 1991). Only the species of the genus *Cystobasidium* (Lagerheim) Neuhoff emend. Yurkov et al. are sensitive to it. A narrow spectrum of the organisms sensitive to them seems to be a characteristic feature of the mycocins exhibiting a fungistatic effect.

It is necessary to emphasize that members of one species are usually homogeneous in their response to mycocins, which is demonstrated by the data on several strains presented in Tables 1–3. The heterogeneity sometimes occurring in this respect is determined, as a rule, by two causes. One of them is associated with cross immunity to immunologically similar mycocins. This is evidenced by frequent heterogeneity among the taxa with known widespread mycocinogeny, for example, among the species of *Hanseniaspora* Zikes, *Ogataea* Yamada et al., and *Wickerhamomyces* (Golubev, 2014, 2015, 2015b, 2015c, 2015d, 2015e). Another cause of a different response to mycocins is the taxonomic heterogeneity of the taxa. For example, variability of *C. boidinii* Ramirez strains in their sensitivity

not only to *W. bovis* mycocin (Table 3), but also to the one produced by *O. nonfermentans* (Wickerham) Kurtzman et Robnett (Golubev, 2015e), is in agreement with their differences in the electrophoregrams of the enzymes, indicating the existence of varieties within this species (Lee and Komagata, 1983).

Different response of *W. canadensis* strains (anamorph–*C. melinii* Diddens et Lodder)—to the synonyms of which *Hansenula wingei* Wickerham was assigned—too *W. bovis* mycocin is of interest (Table 2). This synonymy was based on a considerable (78%) level of DNA homology of these species (Fuson et al., 1979). However, *H. wingei* is heterothallic and forms true mycelium, whereas *W. canadensis* is homothallic and does not form mycelium. Moreover, their type strains do not cross with each other, thus indicating their reproductive isolation (Shchurov et al., 1988).

The species *N. holstii* (Wickerham) Yamada et al. is also heterogeneous in its sensitivity to *W. bovis* mycocin. In contrast to *N. holstii* type strain, *C. silvicola* Shifrine et Phaff 1282 type strain is not sensitive to the mycocin (Table 3). Based on phenotypic similarity, this *Candida* species was regarded as an *N. holstii* anamorph, but the differences revealed in mycocin sensitivity testify to the necessity for further in-depth study of the interrelationship between this teleomorph and anamorph.

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