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EXPERIMENTAL ARTICLES

Antifungal Activity of *Wickerhamomyces silvicola*

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Abstract—*Wickerhamomyces silvicola* strain VKM Y-178 was shown to secrete a mycocin with a fungicidal effect. It exhibits the highest activity at pH 4.5 and elevated osmotic pressure. Over 140 species belonging to 45 genera of ascomycetous yeasts were sensitive to the mycocin, while basidiomycetous species were resistant. Taxonomically homogeneous species usually exhibit a homogeneous response to mycocins.

Keywords: yeasts, mycocinogeny, killer toxin, taxonomy

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Secretion of antifungal (glyco)peptides termed mycocins, or killer toxins, is a common phenomenon in yeast fungi (Golubev, 2012); it has been described in several species of the genus *Wickerhamomyces* Kurtzman et al., in particular, in *W. anomalus* (Hansen) Kurtzman et al. (= *Hansenula anomala* (Hansen) H. et P. Sydow = *Pichia anomala* (Hansen) Kurtzman). Application of mycocinogenic cultures to type candidosis-causing agents and to suppress the growth of fungi spoiling animal feed and alimentary products was proposed (Passoth et al., 2011; Druvefors et al., 2005).

Most mycocinogenic strains of *W. anomalus* are active against nearly all species of their own genus (Golubev, 2015). The only resistant strain was the type strain *W. silvicola* (Wickerham) Kurtzman et al. (= *Hansenula silvicola* Wickerham = *Pichia silvicola* (Wickerham) Kurtzman), VKM Y-178, which made us suppose that it possessed mycocinogenic activity. Subsequent experiments confirmed this hypothesis.

The goal of the present work was to analyze the conditions under which this strain exhibits fungicidal activity, as well as to investigate the range of activity and other characteristics of the secreted mycocin.

MATERIALS AND METHODS

All strains used in the study are maintained in the All-Russian Collection of Microorganisms (http://www.vkm.ru); for this reason, the initialization before the strain number (VKM Y-) is omitted in the text that follows.

To test the stains' sensitivity to the mycocin, the culture in question was grown for 2–3 days on malt agar, and 0.05 mL cell suspension (~ 10^5 cells/mL) was seeded onto glucose–pepton agar (g/L): glucose, 5; pepton, 2.5, yeast extract, 2; agar, 20, and thoroughly dispersed with a spatula. The plate was then streaked with a heavy inoculum of a 3- to 4-day culture of the toxin-producing strain 178. The seeded plates were incubated at room temperature until the appearance of the lawn. The lawn-forming culture was classified as sensitive if strain 178 suppressed its growth in a zone several millimeters wide; if the zone of suppressed growth was up to 1–2 mm wide, the fungal strain was considered as weakly sensitive. Strains for which no growth suppression zone was observed were classified as insensitive.

To produce the extracellular toxin, strain 178 was grown for 3 days in liquid glucose–pepton medium of the same composition in the static mode. The cells were removed by centrifugation at 5000 *g* for 10 min, and the supernatant was filtered through GF/A glass filters (Sigma, the United States). The toxin-containing culture filtrate was used to test the toxin sensitivity to heating . The effect of the toxin on the viability of sensitive cells was evaluated by incubating them in the culture liquid and subsequently plating them on malt agar and counting CFU numbers.

To estimate the molecular weight of the toxin, strain 178 was grown on the medium used for sensitivity tests on agar plates covered with a dialysis membrane (Spectrum, the United States). After 7 days of incubation, the membrane with the strain 178 culture was removed from the solid medium, and the plates were seeded with the toxin-sensitive strain *W. canadensis* 1395.

To test whether the antifungal activity could be eliminated, suspension of strain 178 cells (0.1 mL, 10^4 cells/mL) was plated on malt agar and incubated at the maximum temperature supporting its growth. Alternatively, the plates were irradiated with UV light (254 nm, ~200 J/m^2) for 3–6 min.

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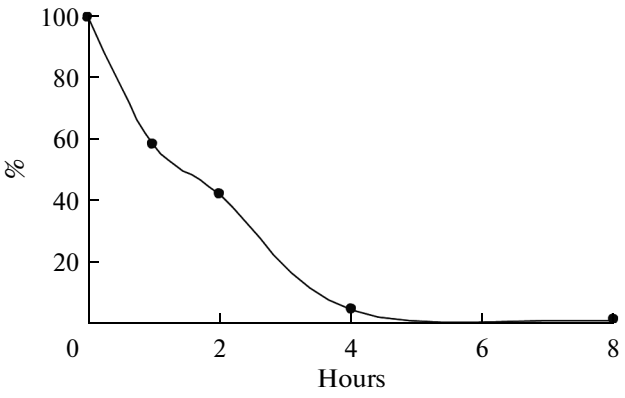
RESULTS AND DISCUSSION

Strain 178 exhibited antifungal activity at pH values ranging from 3.5 to 6.0. At the end points of this range, the activity was weak, and it was absent at pH 6.5 or higher. The broadest zones of growth suppression in sensitive cultures were obtained at pH 4.5. Their width increased considerably if the medium was supplemented with 3% NaCl, and increased even more in the presence of 5% NaCl. However, in the latter case, growth of the culture was strongly delayed; for this reason, the sensitivity of yeast species to the agent produced by *W. silvicola* 178 was studied using glucose–pepton agar on citrate–phosphate buffer (pH 4.5) or supplemented with 3% NaCl. It should be noted that another *W. silvicola* strain, 2191, described as *Endomycopsis muscicola* Nakase et Komagata, did not exhibit antifungal activity and, moreover, it was sensitive to the agent secreted by strain 178. These two strains may be useful for identification of the genes responsible for the synthesis of the mycocin.

The toxin secreted by strain 178 was completely inactivated after heating to 100°C for 5 min. It did not diffuse through the membrane impermeable for compounds with a molecular weight of 8 kDa and above. This compound exhibited fungicidal activity: approximately a half of sensitive cells died after 1–2 h of incubation with this agent, and nearly all them died in 4–5 h (figure).

Apparently, the synthesis of the antifungal toxin in strain 178 is determined by chromosomal genes, since it was not affected by treatments eliminating the extrachromosomal elements: none of the more than 120 randomly selected colonies lost its activity after the strain had been incubated at the maximum temperature supporting its growth (35°C) or subjected to UV irradiation.



Killing of *Wickerhamomyces canadensis* 1395 cells (% viable) incubated in a mycocin-containing culture liquid of *Wickerhamomyces silvicola* 178.

The effect of the agent secreted by the *W. silvicola* strain was tested in over 220 yeast species representing 86 genera. Their sensitivity to the toxin was taxon-specific. The toxin was not active against *Schizosaccharomyces pombe* (*Schizosaccharomycetes*), nor against strains representing 23 genera of basidiomycetous yeasts belonging to *Agaricostilbomycetes, Cystobasidiomycetes, Exobasidiomycetes, Macrobotryomycetes*, and *Tremellomycetes* (Table 1), but only against ascomycetous yeasts of the class *Saccharomycetes.* Based on the sum of traits characterizing the antifungal agent produced by *W. silvicola* strain 178, such as taxonomic specificity of its killing pattern, thermolability, considerable molecular weight, and the conditions for

Table 1. Genera whose species were insensitive to the mycocin from *Wickerhamomyces silvicola* (number of species and strains tested)

Aciculoconidium (1, 1)	*Filobasidium* (1, 1)	*Pachysolen* (1, 1)
Ambrosiozyma (1, 1)	*Holtermannia* (1, 1)	*Priceomyces* (1, 1)
Babjeviella (1, 1)	*Hyphopichia* (1, 1)	*Rhodosporidium* (1, 1)
Blastobotrys (1, 1)	*Kloeckera* (1, 1)	*Rhodotorula* (1, 1)
Bulleromyces (1, 1)	*Kockovaella* (1, 1)	*Sakaguchia* (1, 1)
Citeromyces (1, 1)	*Kodamaea* (1, 1)	*Schizosaccharomyces* (1, 2)
Cyptococcus (1, 1)	*Komagataella* (1, 1)	*Sirobasidium* (1, 1)
Curvibasidium (1, 1)	*Kondoa* (1, 1)	*Sporidiobolus* (1, 1)
Cystofilobasidium (1, 1)	*Kurtzmanomyces* (1, 1)	*Sympodiomycopsis* (1, 1)
Dioszegia (1, 1)	*Kwoniella* (1, 1)	*Tolulaspora* (3, 3)*
Eremothecium (1, 1)	*Leucosporidium* (2, 2)	*Trimorphomyces* (1, 1)
Fellomyces (1, 1)	*Magnusiomyces* (1, 1)	*Wickerhamiella* (1, 1)
Fibulobasidium (1, 1)	*Millerozyma* (1, 1)	*Yamadazyma* (1, 1)
Filobasidiella (2, 2)	*Mrakia* (1, 1)	

* *T. delbrueckii* (1) was weakly sensitive.

Table 2. Spectrum of action of *Wickerhamomyces silvicola* mycocin among representatives of the *Wickerhamomycetaceae*, including phylogenetically related *Candida* species

Barnettozyma californica 838T, 2553	+	*Cyb. veronae* 2163	+
B. pratensis 2136T	+	*Starmera amethionina* 2614T, 2615	+
B. salictaria 1288T	+	*S. pachycereana* 2616T, 2617	+
Candida odintsovae 2024–2027	+	*W. alni* 2509T, 2510	+
C. silvicultrix 2189T	+	*W. anomalus** 1086T	–
C. quercuum 2157T, 2739	+	*W. bisporus* 1065T	+
C. solani 69T, 2334, 2335	+	*W. bovis* 1106T	+
Cyberlindnera americana 1409T	+	*W. canadensis* 1395T, 1398–1400	+
Cyb. bimundalis 1407T	+	*W. chambardii* 276	+
Cyb. fabianii 1450T	+	*W. ciferri* 169T	–
Cyb. jadinii 768	+	*W. lynferdii* 2205T	+
Cyb. mrakii 173T	–	*W. mucosus* 2086T	–
Cyb. petersonii 1410	+	*W. pijperi* 310T	+
Cyb. rhodanensis 277	+	*W. rabaulensis* 2197T	+
Cyb. sargentensis 2087T	+	*W. silvicola* 2191	+
Cyb. saturnus 2552HT	–	*W. strasburgensis* 278, 1387, 2746	+
1084, 1403, 2551	w	*W. subpelliculosus* 180T	+
Cyb. suaveolens 2990T	+	1091	w
Cyb. subsufficiens 2220T	w	1085, 1088, 1089, 1090	–
		W. sydowiorum 2192	+

Here and Tables 3–7: +, sensitive; –, insensitive; w, weakly sensitive. T, type strain; NT, neotype strain. 13 strains were weakly sensitive, the other 37 were insensitive.

expression of its activity, it can be classified as a mycocin (Golubev, 2012).

The *W. silvicola* mycocin had a fairly broad spectrum of action, which included over 140 species of 45 genera of ascomycetous yeasts of the order *Saccharomycetales*. Nearly all members of the family *Wickerhamomycetaceae* were sensitive to the toxin (Table 2). In other families, especially phylogenetically remote ones, the number of sensitive species was lower and the number of weakly sensitive was higher (Table 3–7).

Strains of the same species usually exhibited a uniform reaction to the mycocin from *W. silvicola* (e.g., *B. californica, C. odintsovae, C. parapsilosis, C. quercuum, C. solani, L. kononenkoe, L. lipofer, M. guilliermondii, N. commutata, N. fulvescens, P. norvegensis, St. amethionina, W. canadensis*, and *W. strasburgensis*). However, quite a few taxa were heterogeneous in this respect (*Cyb. saturnus, L. thermotolerans, L. starkeyi, L. tetrasporus, P. fermentans, P. kluyveri, P. manshurica, P. membranifaciens, S. starkeyi-henricii, W. anomalus, W. subpelliculosus*), which may be due to different reasons (Tables 2–7). In particular, such heterogeneity can be associated with cross-immunity to similar mycocins produced by related species, as is apparently the case for *W. anomalus* strains (Table 2).

However, in most cases this heterogeneity was suggestive of a taxonomic one. It is worth noting that heterogeneous reactions to the mycocin were mainly observed for the taxa that included species whose names are considered as synonyms (with their type strains). For instance, the neotype strain *Cyb. saturnus* (Klöcker) Minter was mycocin-insensitive, in contrast to strains 1403 and 2551, type strains of *Hansenula coprophila* Soneda and *H. beijerinckii* van der Walt, currently considered as synonymic (Table 2). Another example of such a case is *Saccharomyces veronae* Lodder et Kreger-van Rij, whose two strains, including type strain 533, were mycocin-resistant, in contrast to

Table 3. Spectrum of action of *Wickerhamomyces silvicola* mycocin among representatives of the *Debaryomycetaceae* and *Metschnikowiaceae*, including phylogenetically related *Candida* species

Debaryomycetaceae		*S. etchellsii* 1391T	+
Candida albicans 2994NT	+	*S. occidentalis* 673T	+
C. parapsilosis 58T, 1224	+	*S. polymorphus* 1392T	–
C. tropicalis 2771T	w	*S. pseudopolymorphus* 311	w
C. viswanathii 1491T	+	*S. vanrijiae* 2627T	+
Debaryomyces coudertii 2570	+	*Wickerhamia fluorescens* 1465T	w
D. fabryi 102T	+	Metschnikowiaceae	
D. hansenii 116T	+	*Clavispora lusitaniae* 1022	+
D. maramus 100	–	*Metschnikowia agaves* 2840T	–
D. nepalensis 2571T	+	*M. australis* 2670T	+
D. robertsiae 1500T	+	*M. bicuspidata* 2628HT	+
D. subglobosus 106T	*w*	*M. gruessii* 2	w
Lodderomyces elongisporus 426T	+	*M. krissii* 220T	+
Meyerozyma guilliermondii 41, 1256T	+	*M. lunata* 1651T	+
Scheffersomyces spartinae 2144T	w	*M. pulcherrima* 64	w
S. stipitis 2160T	+	*M. reukaufii* 1466	+
Schwanniomyces capriottii 1652T	+	*M. zobellii* 221T	+

+, sensitive; –, insensitive; w, weakly sensitive.

Table 4. Spectrum of action of *Wickerhamomyces silvicola* mycocin among representatives of the *Pichiaceae*, *Saccharomycodaceae*, and *Saccharomycopsidaceae*

Pichiaceae		*Saturnispora dispora* 418T	–
Dekkera anomala 19	+	*S. saitoi* 315	+
D. bruxellensis 21	+	Saccharomycodaceae	
Kregervanrija delftensis 1393T	+	*Hanseniaspora guilliermondii* 136T	w
K. fluxuum 1285T	+	*H. occidentalis* 211T	w
Pichia besseyi 2084	–	*H. osmophila* 214T	–
P. deserticola 246T	+	*H. uvarum* 215T	–
P. fermentans 244, 296T	w	*H. valbyensis* 138T	w
1518	–	*H. vinae* 197	–
P. kluyveri 290	–	*Saccharomycodes ludwigii* 626	+
297T	w	Saccharomycopsidaceae	
P. manshurica 298T	w	*Saccharomycopsis capsularis* 2077T	+
312A	–	*S. crategensis* 2210T	–
P. membranifaciens 288	+	*S. fermentans* 2853T	+
248, 292, 299T, 2648	–	*S. fibuligera* 1067T	–
1105	w	*S. javanensis* 1069T	–
P. nakasei 2070	w	*S. malanga* 2212T	–
P. norvegensis 71, 1512, 2009	+	*S. schoenii* 1073T	+
P. occidentalis 1461	w	*S. selenospora* 131T	w
P. terricola 317	+	*S. synnaedendra* 2673T	–
		S. vini 2089T	–

+, sensitive; –, insensitive; w, weakly sensitive.

Table 5. Spectrum of action of *Wickerhamomyces silvicola* mycocin among representatives of the *Saccharomycetaceae,* including phylogenetically related *Candida* species

Candida glabrata 1481T	+	*Lachancea cidri* 2145T	+
C. holmii 738T	–	*L. fermentati* 862T	–
C. kefyr 257T	+	*L. kluyveri* 1295T	–
C. milleri 2185T	w	*L. thermotolerans* 533, 534	–
C. spherica 762T	+	694, 894T, 2317	+
Kazachstania africana 216	+	*Nakaseomyces delphensis* 1529T	w
K. barnettii 2843T	–	*Naumovozyma dairenensis* 412T	w
K. bovina 1021T	+	*Saccharomyces bayanus* 349T	+
K. exigua 1147HT	w	*S. cariocanus* 2878T	–
K. lodderae 478	+	*S. cerevisiae* 375HT	–
K. rosini 2849	–	*S. kudriavzevii* 2880T	–
K. slooffiae 2104T	w	*S. mikatae* 2882T	+
K. spenserorum 2847T	+	*S. paradoxus* 483T	–
K. transvaalensis 521	+	*S. pastorianus* 507HT	+
K. unispora 597T	+	*Tetrapisispora blattae* 2216T	w
K. viticola 1659T	+	*Vanderwaltozyma polyspora* 1524T	+
K. yakushimaensis 2846T	+	*Zygosaccharomyces bailii* 850T	+
Kluyveromyces aestuarii 1528T	w	*Z. bisporus* 853T	w
Kl. dobzhanskii 1293T	+	*Z. kombuchaensis* 2920	w
Kl. lactis 830, 868HT, 1302	+	*Z. mellis* 877T	w
Kl. marxianus 876T	+	*Z. rouxii* 866T	w
Kl. wickerhamii 1297T	+	*Zygotorulaspora mrakii* 2198T	w

+, sensitive; –, insensitive; w, weakly sensitive.

Table 6. Spectrum of action of *Wickerhamomyces silvicola* mycocin among representatives of the *Lipomycetaceae*, *Dipodascaceae*, and *Trichomonoascacea*, including phylogenetically related *Geotrichum* and *Myxozyma* species

Dipodascaceae			
Geotrichum fermentans 813T	+	*L. tetrasporus* 1413, 2489, 2490	w
Lipomycetaceae		1968, 2488	–
Dipodascopsis anomalus 1969T	w	*Myxozyma melibiosi* 2446T	–
Lipomyces lipofer 218T, 1094,		*M. mucaligina* 2714T	w
1415, 2485, 2669	–	Trichomonoascaceae	
L. kononenkoae 1997, 2105,		*Sugiyamaella smithiae* 1449	w
2715T	w	*Zygoascus hellenicus* 2611T	–
L. starkeyi 219T, 2487	–	*Z. tannicolus* 2150	+
1414, 2486	w		

+, sensitive; –, insensitive; w, weakly sensitive.

Table 7. Spectrum of action of *Wickerhamomyces silvicola* mycocin among yeast fungi of an uncertain phylogenetic position

Kuraishia capsulata 2077T	+	*Og. parapolymorpha* 2518	+
Nadsonia commutata 1573T,		*Og. philodendra* 2168T	+
1941–1946, 2610	+	*Og. pini* 886T	+
N. fulvescens 268–270, 1653,		*Og. polymorpha* 2559T	+
1923–1926, 2618, 2530,		*Og. trehalophila* 1289T	–
2578, 2579, 2531, 2532T	–	*Og. wickerhamii* 2629T	+
Nakazawaea holstii 2558T	+	*Peterozyma toletana* 2151T	+
Ogataea glucozyma 2079	+	*Schizoblastosporion starkeyihenricii* 1998,	
Og. henricii 2080	–	2534, 2535, 2762T	w
Og. methanolica 2621	–	1947–1949, 1999, 2536–2542	–
Og. methylovora 2581T	–	*Trigonopsis variabilis* 2601T	+
Og. minuta 172T	+	*Sporopachydermia lactativora* 1647	+
Og. naganishii 2161T	–	*Yarrowia deformans* 1444T	+
Og. nonfermentans 2081T	+	*Yar. lipolytica* 47	–

+, sensitive; –, insensitive; w, weakly sensitive.

other strains, together with the type strain of *L. thermotolerans* (Table 5). The situation concerning *W. subpelliculosus* is rather controversial (Table 2). Previously, this species was classified into the genera *Hansenula*, and then to *Pichia*, and different strains were considered as type ones.

Heterogeneous response to the *W. silvicola* mycocin was particularly strongly pronounced among species of the genus *Pichia* (Table 4), which are also characterized with especially large number of synonyms. These data correlate with the results of our previous work where mycocinotyping was used to demonstrate taxonomic heterogeneity of *P. membranifaciens* (Golubev and Blagodatskaya, 1993); on the whole, it suggests that this method is an efficient means of evaluating the homogeneity of yeast taxons.

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