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EXPERIMENTAL ARTICLES

Intraspecific and Intrageneric Antagonistic Activity of *Wickerhamomyces anomalus*

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Abstract—A total of 53 *Wickerhamomyces anomalus* strains were examined for antagonistic activity, including the nomenclature types of the species with the names presently considered synonymous. Over 70% of the strains exhibited antibiotic activity. According to the action spectra of intraspecific activity, the strains fell into three groups, while according to their activity against other *Wickerhamomyces* species and phylogenetically related *Candida* species they formed five subgroups. Antibiotic agents (mostly mycocins) varied in their physicochemical properties

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The yeasts *Wickerhamomyces anomalus* (Hansen) Kurtzman et al. [=*Hansenula anomala* (Hansen) H. et P. Sydow = *Pichia anomala* (Hansen) Kurtzman] have been found practically worldwide in a variety of substrates. This fact, together with their considerable phenotypic variability [1], differences in the chromosome number (9 to 12), chromosome size (850–3500 kb) [2], and the β-tubulin gene sequences [3] cause doubts in their taxonomic homogeneity [4]. In most cases extensive synonymy of this species is based on the phenotypic similarity. Only *Candida pelliculosa* Redaelli, *H. anomala* (Hansen) H. et P. Sydow var. *schneggii* (Weber) Wickerham, and *H. ukrainica* Kvasnikov et al. were shown to be synonyms of *W. anomalus* by means of DNA–DNA hybridization [5]. While sequencing of D1/D2 regions of rDNA of some strains revealed their similarity to the type strain of the species, conspecificity of the cultures was not confirmed genetically due to low mating and sporulation activity and low ascospore fertility [2].

Being of interest for production of polyols and phytase, *W. anomalus* attracts special attention due to its antibiotic activity. This activity is probably responsible for resistance of its cultures to contamination, which was reported long ago [4] and initiated numerous proposal for application of *W. anomalus* for biotyping and suppression of pathogenic and food-contaminating fungi [6, 7]. In most cases the mechanism of antibiotic activity has not been revealed. Sometimes it is attributed to the competition for nutrient substrates, but more often to production of extracellular metabolites, such as alcohols, acids [4], volatile compounds [8], lytic enzymes [9], or mycocins (killer toxins) [10].

Importantly, single isolates were used in these works. Investigation of activity of numerous (~100) isolates against *Saccharomyces cerevisiae* Meyen ex Hansen and pathogenic *Candida* Berkhout strains revealed high diversity including the entire range from inactive ones to the isolates suppressing growth of all studied cultures [11, 12]. These results show that available data on antibiotic activity of *W. anomalus* are insufficient and its reliable assessment requires investigation based on a sizable and representative sample of the strains.

The present work presents the results of investigation of intraspecific antagonistic relationships between *W. anomalus* cultures, of which many are types or author strains of anamorphic and teleomorphic species with the names presently considered synonymous. Activity of the strains of this species against members of the genus *Wickerhamomyces*, as well as against phylogenetically related *Candida* species, was also determined.

MATERIALS AND METHODS

Strains from All-Russian Collection of Microorganisms (VKM) [http://www.vkm.ru] were used in the present work. Sensitivity of three-day cultures grown on malt agar was determined at room temperature using the "culture against culture" method. Water suspensions (0.05 mL, 10^5 cells/mL) were spread over the surface of a buffered (with citrate–phosphate buffer) medium containing the following (g/L): glucose, 5.0; peptone, 2.5; yeast extract, 2.0; agar, 20.0. The cul-

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tures of *W. anomalus* were then streak-inoculated. The plates were incubated until cell lawns developed. The strains forming growth inhibition zones several mm wide, zones ~1 mm wide, and no zones were recorded as sensitive, weakly sensitive, and insensitive, respectively.

RESULTS AND DISCUSSION

W. anomalus cultures exhibited antagonistic activity at pH 3.5 to 6.5. At pH 5.0, growth inhibition zones were the most pronounced. They became broader on the media supplemented with NaCl (1–7%) or glycerol (50, 100 mL/L). With few exceptions, both additives yielded similar results. However, *W. strasburgensis* (Ramirez et Boidin) Kurtzman et al. exhibited sensitivity to the agents secreted by *W. anomalus* strains VKM Y-157 and 158 on the medium with glycerol, but not with NaCl, while *W. pijperi* (van der Walt et Tscheuschner) Kurtzman et al. was sensitive to the agents of strains VKM Y-161–163 and 174 on the medium with NaCl, but not with glycerol. The studied strains were examined on both the medium with 3% NaCl and the medium with 50 mL/L glycerol (pH 5.0).

Cross-testing of 53 *W. anomalus* strains revealed six which were weakly sensitive. Considering that producers were immune to their agents, the strains of this species formed three groups depending on their antagonistic reactions. Thus, 28% exhibited no antagonistic activity, while others (72%) formed two groups: six strains (11%) were active against three sensitive cultures, while 32 strains (60%), including the type strain, were active against all six sensitive cultures (Table 1). Blue edging developing on the lawns of sensitive strains along the streaks of group II and group III strains on the medium with methylene blue (0.03 g/L) indicated fungicidal action of secreted agents.

Three intraspecific groups differing in their activity against *Wickerhamomyces* species and closely related *Candida* species fell into five subgroups, of which two comprised members of group I, the fourth contained members of group II, and the third and fifth ones contained members of group III (Table 2).

Ten strains of subgroup I-1 exhibited no antibiotic activity. It includes the type strains *C. pelliculosa* VKM Y-60, *C. pelliculosa* Redaelli var. *cylindrical* Diddens et Lodder VKM Y-61, *H. anomala* (Hansen) H. et P. Sydow var. *sphaerica* (von Nägeli) Dekker VKM Y-156, *Monilia javanica* Went et Geerligs VKM Y-225, and the authentic strain *Endoblastoderma pulverulentum* Fischer et Brebeck VKM Y-118. The strains of subgrouop I-2 were active only against *W. canadensis* (Wickerham) Kurtzman et al., *W. strasburgensis*, and *C. odintsovae* Babjeva et al. On the lawns of the latter strain, no blue edging was formed along the streaks of the strains of this group on the medium with methylene blue.

Table 1. Grouping of *Wickerhamomyces anomalus* strains according to intraspecific cross-testing

Groups	Strains VKM Y-	Sensitive strains, VKM Y-	
		146 150 152	164 2353 2354
I	60, 61, 118, 146, 156–158, 164, 225, 1908–1910, 2353, 2354, 2511	–	–
II	148, 150, 152, 153, 2512, 2513	w	–
III	140–145, 147, 149, 151, 154, 155, 159–163, 170, 171, 174, 175, 177, 1086^T, 1087, 1431, 1905–1907, 2037–2041	w	w

"+," "w," and "–" stand for sensitive, weakly sensitive, and insensitive, respectively. T indicates the type strain.

Members of group II, which contained the type strain *H. anomala* (Hansen) H. et P. Sydow var. *longa* Dekker VKM Y-153, were active against ten species—not only *Wickerhamomyces* spp., but also *C. odintsovae* and *C. silvicultrix* van der Walt et al.

The cultures of subgroup III-4 suppressed growth of almost all studied *Wickerhamomyces* strains, except for *W. mucosus* (Wickerham et Kurtzman) Kurtzman et al., *W. pijperi*, and *W. silvicola* (Wickerham) Kurtzman et al. (Table 2). This subgroup included the type strains *H. javanica* Groenewege VKM Y-170 and *Willia productive* Berkhout VKM Y-154. Only *W. silvicola* was insensitive to antibiotic factors secreted by the cultures of subgroup III-5. This subgroup contained type strains *C. beverwijkii* Novak et Vitez VKM Y-1431, *H. anomala* (Hansen) H. et P. Sydow var. *robusta* Dekker VKM Y-155, *H. nivea* Castelli VKM Y-174, *H. panis* Castelli VKM Y-175, *H. ukrainica* VKM Y-2037, *Saccharomyces aceris-sacchari* Fabian et Hall VKM Y-163, *W. anomalus* VKM Y-1086, and *W. schneggii* Weber VKM Y-177.

Interestingly, strain numbers within the groups were close to each other, which indicated their arrival to the collection at a same period and often from the same sources (Table 2). Thus, strains *W. anomalus* VKM Y-157, 158, 1908–1910 (subgroup I-2) and VKM Y-140–144, 1905–1907 (subgroup III-5) were isolated in the Far East from spontaneously fermented plant juices, while strains VKM Y-2037–2041 (subgroup III-5) were isolated from oak leaves in Ukraine. Association of producers of antibiotic agents to specific regions and substrates may indicate that specific agents may act as characteristic markers for *W. anomalus* natural populations.

It should be noted that the most phylogenetically closely related *Wickerhamomyces* and *Candida* species forming a single subcluster [5] often possess identical or very similar sensitivity types. This applies, for exam-

Table 2. Activity of *Wickerhamomyces anomalus* strains against members of this genus and related *Candida* species

Species, strains VKM Y-	*W. anomalus* strains, VKM Y-				
	60 61 118 146 156 164 225 2353 2354 2511	157 158 1908 1909 1910	148 150 152 153 2512 2513	154 160 170	140–145, 147, 149, 151, 155, 159, 161–163, 171, 174, 175, 177, 1086, 1087, 1431, 1905–1907, 2037–2041
	I-1	I-2	II-3	III-4	III-5
Wickerhamomyces canadensis 1395^T	–	+	+	+	+
W. bisporus 1065^T	–	w	+	+	+
W. strasburgensis 278, 1387	–	w	+	+	+
Candida odintsovae 2024–2027	–	w	+	+	+
W. rabaulensis 2197^T	–	–	+	+	+
W. lynferdii 2205^T	–	–	+	+	+
W. sydowiorum 2192	–	–	+	+	+
C. silvicultrix 2189^T	–	–	w	+	+
W. chambardii 276	–	–	w	+	+
W. alni 2509^T, 2510	–	–	w	+	+
C. quercuum 2157^T, 2739	–	–	–	+	+
W. bovis 1106^T	–	–	–	+	+
W. subpelliculosus 180^T	–	–	–	+	+
C. solani 69^T	–	–	–	w	+
W. ciferri 169^T	–	–	–	w	w
W. pijperi 310^T	–	–	–	–	w
W. mucosus 2086^T	–	–	–	–	w
W. silvicola 178^T	–	–	–	–	–

"+," "w," and "–" stand for sensitive, weakly sensitive, and insensitive, respectively. T indicates the type strain.

ple, to the pairs of species *W. lynferdii* (van der Walt et Johannsen) Kurtzman et al.–*W. sydowiorum* (Scott et van der Walt) Kurtzman et al., *W. bisporus* (Beck) Kurtzman–*W. canadensis*, and *W. rabaulensis* Soneda et Uchida–*C. odintsovae* (Table 2).

The nature of antifungal agents secreted by *W. anomalus* strains is known only in some cases. Thus, in members of group III [VKM Y-151, 159, and 1086 (=NCYC 432)] they are glycoproteins identified as mycocins (killer toxins) [7, 13, 14]. Mycocins are probably produced by some other strains studied, likely belonging to group III and most easily detected due to their relatively high activity and broad action spectra [10, 15, 16]. None of these strains is known to contain plasmids, i.e., synthesis of mycocins (with β-1,6-glucan as a receptor) is determined by chromosomal genes. It should be noted, however, that even within a single group the properties of mycocins of different *W. anomalus* strains vary significantly in molecular mass (43 to 300 kDa), thermostability, glycosylation degree (14 to 51%), amino acid composition, and effect of elevated osmotic pressure on their activity.

It should be stressed that, apart from mycocins, other agents may be responsible for the antifungal activity of yeasts [17]. Thus, *W. anomalus* produces sophorolipids [18], while one of the two toxins secreted by strain VKM Y-159, with its heat stability, insensitivity to proteases, and low molecular mass (below 10 kDa) was recently shown [7] to be probably a glycolipid.

Variability of *W. anomalus* in their physiological characteristics, karyotypes, and (as may be seen from the data presented above) in the antifungal agents produced indicates either high variability within this spe-

cies or its taxonomic heterogeneity. In any case, the antibiotic potential of this taxon is more considerable and diverse than was believed previously.

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