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BIOTYPING OF MICELIAL FUNGUS CULTURES BY THE KILLER SYSTEM

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Key words: Hyphomycetes - Killer yeasts - Biotyping.

Based on the occurrence of the yeast killer phenomenon in hyphomycetes, the toxic effect of 37 selected killer yeasts was studied on eleven strains of *Pseudallescheria boydii*, six strains of *Aspergillus niger*, 18 strains of *Penicillium camemberti* and nine strains of *Sporothrix schenckii*. The demonstration of different biotypes within the species of *P. boydii* and *P. camemberti* proves that the killer system also is a practical and effective method for epidemiological studies among hyphomycetes. Based on the system used, it was not possible to observe markedly different biotypes among the *A. niger* and *S. schenckii* strains studied. The first evidence that an isolated, concentrated (50X) and partially purified yeast killer toxin may display a lethal activity against mycelial fungus cultures is also given in this paper.

INTRODUCTION

Gross morphology and microscopic features are inconsistent criteria for differentiating strains of hyphomycetes within the species. Pleomorphism and lack of sporulation pose major difficulties to mycologists attempting the biotyping of mycelial fungi. Recently, monoclonal antibodies were proven useful for serotyping mycelial fungi (1, 5, 7). This immunological approach, however, requires the availability of proper reagents and technology uncommon to small laboratories.

The recent observation that killer yeasts may display an inhibitory effect on different species of hyphomycetes (6) prompted us to evaluate whether the killer system, previously applied to opportunistic yeasts (2-4), could also be routinely and practically used for epidemiological investigation among hyphomycetes.

MATERIALS AND METHODS

Cultures. - The mycelial cultures of *Pseudallescheria boydii, Aspergillus niger, Penicillium camemberti* and *Sporothrix schenckii* used in this study are listed in Table 1. They were isolated from soil or graciously furnished by public sources and maintained in our collection in sterile distilled water. The 37 killer yeasts tested were also withdrawn from our fungus collection. All of them had been previously recognized to show toxic activity on various sensitive strains (Table 2). Among the 37 killer yeasts studied, one (*Hansenula anomala* UCSC 25F) was selected according to its wide spread activity for producing killer toxin; as sensitive fungus isolate was selected *P. boydii* UCSC 7 according to its sensitivity to the killer yeast.

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TABLE 1.
Mycelial fungus cultures investigated by the killer system.

Species	Collection number
Aspergillus niger	UCSC [a] 5-7, 9, 17, 18
Penicillium camemberti	IMI [b] 285506
	IMI 285507
	FRISVAD [c] XX102
	PARIS [d] PCM
	PARIS P9
	PARIS RAPIDEX
	PARIS VB
	PARIS BLANCHE
	PARIS NEIGE
	PARIS STANDARD EXTRA
	PARIS C1
	PARIS C2
	PARIS C3
	PARIS R
	PARIS B5
	PARIS SAM
	FRISVAD DK
	FRISVAD FRR 2170
Pseudallescheria boydii	UCSC 0, 2-11
Sporothrix schenckii	CBS [e] 93072
	UCSC 2
	CBS 30273
	CBS 34035
	CDC [f] B2473
	CDC B2901
	CDC B2560
	CDC B3098
	CDC 2688

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TABLE 2.
Recognized killer yeasts tested against the mycelial fungus cultures listed in Table 1.

Code	Species	Collection number
K 1	*Hansenula* sp.	STUMM [a] 1034
K 2	*Pichia* sp.	STUMM 1035
K 3	*Hansenula anomala*	UM [b] 3
K 4	*H. anomala*	CBS [c] 5759
K 5	*H. anomala*	AHEARN [d] UM 866
K 6	*H. californica*	AHEARN WC 40
K 7	*H. canadensis*	AHEARN WC 41
K 8	*H. dimennae*	AHEARN WC 44
K 9	*H. mrakii*	AHEARN WC 51
K10	*Pichia kluyveri*	STUMM 1002
K11	*Hansenula anomala*	UT [e] 12
K12	*H. bimundalis*	AHEARN WC 38
K13	*H. fabianii*	CBS 5640
K14	*H. petersonii*	AHEARN WC 53
K15	*Pichia guilliermondii*	UT 19
K16	*Saccharomyces cerevisiae*	CDC [f] B2210
K17	*Hansenula bimundalis*	CBS 5642
K18	*H. fabianii*	AHEARN WC 45
K19	*H. holstii*	CBS 4140
K20	*H. subpelliculosa*	CBS 5767
K21	*Pichia ohmeri*	CBS 5367
K22	*Candida guilliermondii*	UCSC [g] 0
K23	*C. maltosa*	UCSC 0
K24	*Pichia spartiniae*	UCSC 0
K25	*Hansenula nonfermentans*	UM 200
K26	*Pichia carsonii*	CBS 810
K27	*P. farinosa*	CBS 185
K28	*P. guilliermondii*	CBS 2031
K29	*Candida pseudotropicalis*	UP [h] 241
K30	*C. pseudotropicalis*	UP 254
K31	*C. pseudotropicalis*	UP 330
K32	*Pichia kluyveri*	UP 5F
K33	*P. kluyveri*	UP 6F
K34	*P. membranafaciens*	UP 10F
K35	*P. kluyveri*	UP 11F
K36	*Hansenula anomala*	UCSC 25F
K37	*H. mrakii*	UCSC 255

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Media. - The original medium, previously adopted for the differentiation of opportunistic yeasts (2, 3), (Sabouraud modified agar, Difco Laboratories, buffered at pH 4.5 with 0.1 M citric acid and 0.2 M potassium phosphate dibasic anhydrous added with 0.03% methylene blue) was used for all the hyphomycetes studied. Sabouraud dextrose agar (Difco Laboratories) was the growth medium for the hyphomycetes and yeasts. Sabouraud broth at pH 3 (same buffer of the solid medium) was used for the production of the isolated killer toxin.

Production of the killer toxin. - One liter flasks with 500 ml of Sabouraud broth (pH 3) were seeded with a loopful of 48-hour-old culture of the selected killer yeast and incubated for 24 h at 24°C in shaken conditions. After this period, the cells were removed by centrifugation, the supernatant was filtered and concentrated 50X

with a PM10 membrane in a TCF10 ultrafiltration cell under nitrogen pressure (Amicon Corporation, Danvers, Ma) (4).

Test performance. - Seven day old mycelial cultures, grown on Sabouraud dextrose agar slants, were scraped to obtain a heavy suspension in distilled sterile water. One ml of the suspension was then mixed with 20 ml of the buffered medium maintained at 45°C and poured into a petri dish. After cooling, 50 µl drops of a heavy distilled water suspension of each killer yeast grown on Sabouraud dextrose agar for 48 h at 25°C were placed on the surface of the agar containing the mycelial isolate to be tested. The plates were incubated at 25°C. The test was repeated four times with different subcultures for each isolate studied. For testing the activity of the concentrated killer toxin, 10 mm diameter wells were punched in the agar and filled with 100 µl of killer toxin.

Reading and interpretation of the results. - The plates were read at different period of time according to the rapidity of growth of the investigated strains: 7 days for *A. niger* and *P. camemberti* and 10 days for *P. boydii* and *S. schenckii*. The killer effect was considered positive when a clear zone of inhibition surrounded the killer colony. A code, previously adopted in our Institute for differentiating opportunistic yeasts (3), was used to record the combined effect of selected killer yeasts and to distinguish sensitive mycelial strains (Table 3).

TABLE 3.
Code number attribution according to the reactions observed in sensitive strains for three consecutive killer strains forming a triplet.

Activity of the killer strains 1st killer	2nd killer	3rd killer	Code number
+	+	+	1
+	+	—	2
+	—	+	3
—	+	+	4
+	—	—	5
—	+	—	6
—	—	+	7
—	—	—	8

RESULTS

Many killer yeasts proved to have a marked toxic effect on the mycelial fungus cultures as expected (Table 4). The *P. boydii* and *S. schenckii* isolates showed a floccose growth with a more or less large zone of inhibition around the killer strains. *A. niger* and *P. camemberti* produced a more velvety growth with marked and narrow lysis.

Group of isolates within the species *P. camemberti* and *P. boydii* showed a different sensitivity

TABLE 4.
Relationships between yeast killer activity and mycelial fungus culture sensitivity.

Sensitive strains	Yeast killer activity *
Aspergillus niger	
UCSC [a] 5	1-9, 11, 21, 29-31, 36, 37
UCSC 6	1-9, 11, 21, 29-31, 36, 37
UCSC 7	1-9, 11, 21, 29-31, 36, 37
UCSC 9	1-9, 11, 21, 29-31, 36, 37
UCSC 17	1-9, 11, 21, 29-31, 36, 37
UCSC 18	1-9, 11, 21, 29-31, 36, 37
Penicillium camemberti	
IMI [b] 285506	2-8, 11, 13, 14, 16, 18, 28-31, 36
IMI 285507	2-8, 11, 28, 36
FRISVAD [c] XX102	2-8, 11, 13, 14, 16, 18, 28-31, 36
PARIS [d] PCM	2-8, 11, 28, 36
PARIS P9	2-8, 11, 13, 14, 16, 18, 28-31, 36
PARIS RAPIDEX	2-8, 11, 13, 14, 16, 18, 28-31, 36
PARIS VB	2-8, 11, 13, 14, 16, 18, 28-31, 36
PARIS BLANCHE	2-8, 11, 13, 14, 16, 18, 28-31, 36
PARIS NEIGE	2-8, 11, 13, 14, 16, 18, 28-31, 36
PARIS STANDARD EXTRA	2-8, 11, 13, 14, 16, 18, 28-31, 36
PARIS C1	2-8, 11, 28, 36
PARIC C2	2-8, 11, 28, 36
PARIS C3	2-8, 11, 13, 14, 16, 18, 28-31, 36
PARIS R	2-8, 11, 13, 14, 16, 18, 28-31, 36
PARIS B5	2-8, 11, 13, 14, 16, 18, 28-31, 36
PARIS SAM	2-8, 11, 13, 14, 16, 18, 28-31, 36
FRISVAD DK	1-9, 11, 20, 21, 23, 28-31, 36, 37
FRISVAD FRR 2170	1-9, 11, 20, 21, 23, 28-31, 36, 37
Pseudallescheria boydii	
UCSC 0	2-8, 11, 28, 36
UCSC 2	2-11, 13, 14, 18, 27, 28, 33-37
UCSC 3	2-8, 11, 36, 37
UCSC 4	2-8, 11, 36
UCSC 5	2-8, 11, 36, 37
UCSC 6	2-8, 11, 23, 28, 36
UCSC 7	2-11, 13-17, 19-22, 24, 31, 33-37
UCSC 8	2-8, 11, 36
UCSC 9	2-9, 11, 21, 28, 33-37
UCSC 10	2-9, 11
UCSC 11	2-8, 11, 36, 37
Sporothrix schenckii	
CBS [e] 93072	1-9, 11, 21, 28-31, 36, 37
UCSC 2	1-9, 11, 21, 28-31, 36, 37
CBS 30273	1-9, 11, 21, 28-31, 36, 37
CBS 34035	1-9, 11, 21, 28-31, 36, 37
CDC [f] B2473	1-9, 11, 21, 28-31, 36, 37
CDC B2901	1-9, 11, 21, 28-31, 36, 37
CDC B2560	1-9, 11, 21, 28-31, 36, 37
CDC B3098	1-9, 11, 21, 28-31, 36, 37
CDC 2688	1-9, 11, 21, 28-31, 36, 37

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* Active killer yeasts are numbered according to the codes given in Table 2. Inactive killer yeasts are not listed.

to the action of the killer yeasts used in this study. For practical purposes, nine killer yeasts could be selected and a code number given to each strain belonging to the species for grouping the isolates into biotypes (Tables 5 and 6).

TABLE 5. — Conventional grouping of *Penicillium camemberti* isolates by the killer system.

Strain number	Killer yeasts arranged in triplet									Code		
	K1	K4	K5	K18	K21	K25	K28	K36	K37			
IMI 285506	—	+	+	+	—	—	+	+	—	4	5	2
IMI 285507	—	+	+	—	—	—	+	+	—	4	8	2
FRISVAD XX102	—	+	+	+	—	—	+	+	—	4	5	2
PARIS PCM	—	+	+	—	—	—	+	+	—	4	8	2
PARIS P9	—	+	+	+	—	—	+	+	—	4	5	2
PARIS RAPIDEX	—	+	+	+	—	—	+	+	—	4	5	2
PARIS VB	—	+	+	+	—	—	+	+	—	4	5	2
PARIS BLANCHE	—	+	+	+	—	—	+	+	—	4	5	2
PARIS NEIGE	—	+	+	+	—	—	+	+	—	4	5	2
PARIS STANDARD EXTRA	—	+	+	+	—	—	+	+	—	4	5	2
PARIS C1	—	+	+	—	—	—	+	+	—	4	8	2
PARIS C2	—	+	+	—	—	—	+	+	—	4	8	2
PARIS C3	—	+	+	+	—	—	+	+	—	4	5	2
PARIS R	—	+	+	+	—	—	+	+	—	4	5	2
PARIS B5	—	+	+	+	—	—	+	+	—	4	5	2
PARIS SAM	—	+	+	+	—	—	+	+	—	4	5	2
FRISDAV DK	+	+	+	—	+	—	+	+	+	1	6	1
FRISDAV FRR 2170	+	+	+	—	+	—	+	+	+	1	6	1

TABLE 6. — Conventional grouping of *Pseudallescheria boydii* isolates by the killer system.

Strain number	Killer yeasts arranged in triplet									Code		
	K5	K9	K10	K20	K31	K33	K34	K35	K36			
UCSC 0	+	—	—	—	—	—	—	—	+	5	8	7
UCSC 2	+	+	+	—	—	+	+	+	+	1	7	1
UCSC 3	+	—	—	—	—	—	—	—	+	5	8	7
UCSC 4	+	—	—	—	—	—	—	—	+	5	8	7
UCSC 5	+	—	—	—	—	—	—	—	+	5	8	7
UCSC 6	+	—	—	—	—	—	—	—	+	5	8	7
UCSC 7	+	+	+	+	+	+	+	+	+	1	1	1
UCSC 8	+	—	—	—	—	—	—	—	+	5	8	7
UCSC 9	+	+	—	—	—	+	+	+	+	2	7	1
UCSC 10	+	+	—	—	—	—	—	—	—	2	8	8
UCSC 11	+	—	—	—	—	—	—	—	+	5	8	7

On the basis of these conventional criteria, the *P. camemberti* isolates could be grouped into 3 different biotypes: 1 6 1 (2 strains), 4 5 2 (12 strains) and 4 8 2 (4 strains). *P. boydii* had 5 biotypes: 1 1 1 (Fig. 1), 1 7 1, 2 7 1, 2 8 8, each characterized by only one strain and 5 8 7 (7 strains) (Fig. 2).

No basic difference in sensitivity among the *A. niger* and *S. schenckii* isolates was observed to justify differentiation into biotypes. The isolated killer toxin proved to produce even a stronger toxic effect on the selected sensitive strain investigated (Fig. 3).

DISCUSSION

The yeast killer effect proved to have widespread and differential activity when tested on several isolates belonging to certain species of mycelial cultures (*P. camemberti* and *P. boydii*). The homogeneous behavior of the isolates of other species (*A. niger* and *S. schenckii*) could be, in our opinion, attributed to the strain characteristics either of the killer yeasts or the sensitive strains used in this study. This is confirmed by the need of considering different killer yeasts for biotyping the *P. camemberti* and *P. boydii* isolates.

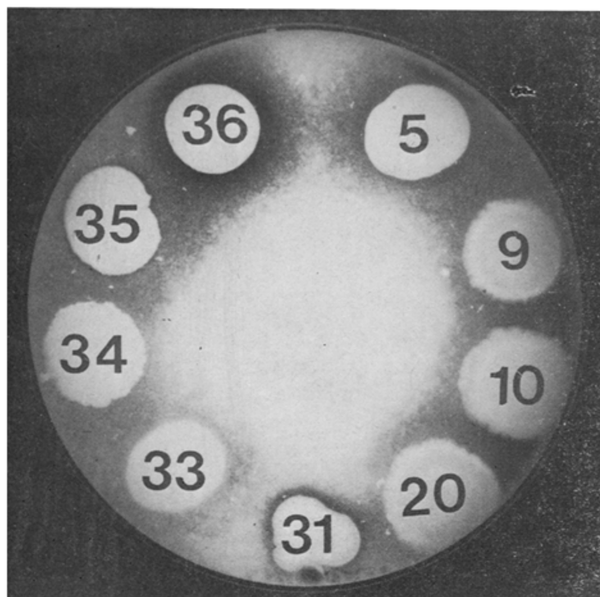

Figure 1. - Differential activity of selected killer yeasts on *Pseudallescheria boydii* UCSC 7.

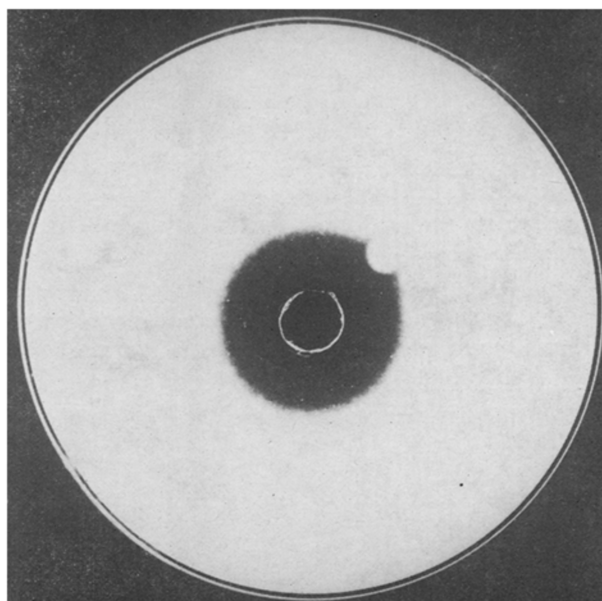

Figure 3. - Effect of the *Hansenula anomala* UCSC 25 F killer toxin on *Pseudallescheria boydii* UCSC 7.

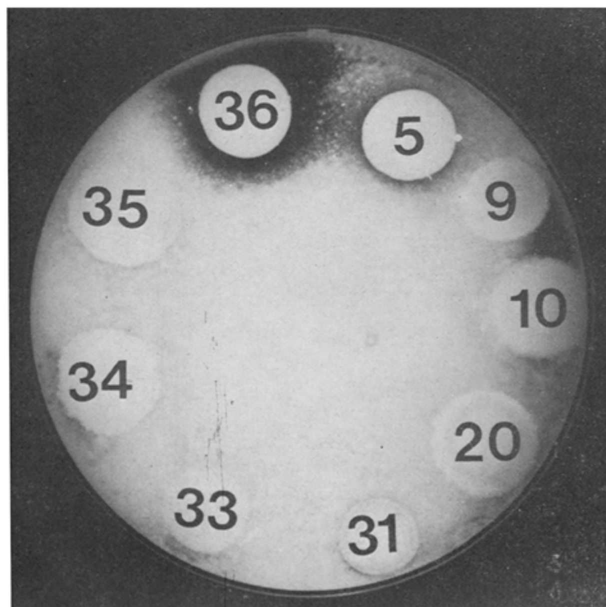

Figure 2. - Differential activity of selected killer yeasts on *Pseudallescheria boydii* UCSC 4.

It is presumed that more biotypes should appear as more killer yeasts and more sensitive strains are investigated. The potential for the biotyping of mycelial fungus cultures by the practical killer system could be applied for epidemiological purposes.

The extension of the method to other pathogenic species could represent a profitable tool for investigating cases of acquired mycoses. The application of the same method to isolates of commercial importance, such as *P. camemberti* in the cheese industry, would permit the easy differentiation of specific strains as well as the control of culture features.

The demonstration of activity of the isolated killer toxin was also of interest. Isolated killer toxin was also of interest. Isolated killer toxins could be presumptively applied also for the differentiation of strains of hyphomycetes as previously shown with the opportunistic yeast *Candida albicans* (4).

The killer system could find profitable application in the biotyping of fungus mycelial cultures in which not many other methods are available as well as yeast fungus cultures. The apparent need of adopting different killer yeasts for biotyping different species of hyphomycetes could be superated by testing a larger group of potential killer yeasts for detecting new relationships between killer and sensitive strains or by using isolated, partially purified killer toxins of predetermined differential activity.

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REFERENCES

1. *Morace G., Amalfitano G.* and *Polonelli L.* (1986): Serotyping of fungal isolates by precipitating monoclonal antibodies. - Mycopathologia, *94:* 53-57.
2. *Morace G., Archibusacci C., Sestito M.* and *Polonelli L.* (1984): Strain differentiation of pathogenic yeasts by the killer system. - Mycopathologia, *84:* 81-85.
3. *Polonelli L., Archibusacci C., Sestito M.* and *Morace G.* (1983): Killer system: a simple method for differentiating srains of *Candida albicans*. - J. Clin. Microbiol., *17:* 774-780.
4. *Polonelli L., Castagnola M., Rossetti D.V.* and *Morace G.* (1985): Use of killer toxins for computer-aided differentiation of *Candida albicans* strains. - Mycopathologia, *91:* 175-179.
5. *Polonelli L.* and *Morace G.* (1985): Serological analysis of dermatophyte isolates with monoclonal antibodies produced against *Microsporum canis*. - J. Clin. Microbiol., *21:* 138-139.
6. *Polonelli L.* and *Morace G.* (1986): Reevaluation of the yeast killer phenomenon. - J. Clin. Microbiol., *24:* 866-869.
7. *Polonelli L., Castagnola M.* and *Morace G.* (1986): Identification and serotyping of *Microsporum canis* isolates by monoclonal antibodies. - J. Clin. Microbiol., *23:* 609-615.