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Reevaluation of the Yeast Killer Phenomenon

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The killer effect of 36 *Hansenula*, *Pichia*, *Saccharomyces*, and *Candida* species on 26 hyphomycetes isolates, 1 isolate of the achlorophyllous microorganism *Prototheca*, 4 isolates of the lipophilic yeast *Malassezia*, 1 isolate of the aerobic actinomycete *Nocardia*, and 19 isolates of bacteria was studied. The killer phenomenon, which was previously considered to be restricted to yeasts, was found to occur among unrelated microorganisms.

The first observation of antagonism in microorganisms was probably reported by Pasteur and Joubert (5). They observed the inhibitory effect on *Bacillus anthracis* of bacteria isolated from urine. A wide range of antimicrobial substances has been successively characterized, including antibiotics, bacteriolytic enzymes, and bacteriocins. In 1963, Bevan and Makower (1) reported, for the first time in yeasts, that a few isolates of *Saccharomyces cerevisiae* secreted a substance that was lethal to other strains of the same species. Since the original discovery of the killer phenomenon in yeasts, several reports have addressed the question of the frequency of occurrence and range of specificity of yeast killer toxins (6, 9). The ability to kill sensitive strains was reported to be widespread in yeasts, although the establishment of killer properties had to await the use of proper screening conditions, which proved to be critical.

On the basis of these reports, we initiated a study of the killer phenomenon in yeasts which permitted the development of a simple system (killer system) useful for differentiating strains of opportunistic yeasts, including *Candida albicans*, within the species (4, 7, 8).

In this report, we present for the first time evidence on the occurrence of sensitivity to yeast killer toxins among other eucaryotic microorganisms and bacteria.

Yeast cultures (K) previously tested for their killer activity on sensitive yeast isolates were received from public and private collections (C. Stumm, University of Njimegen, Njimegen, The Netherlands; UM, Istituto d'Igiene, Università di Milano, Milan, Italy; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; D. G. Ahearn, Georgia State University, Atlanta; UT, Istituto d'Igiene, Università di Torino, Turin, Italy; CDC, Centers for Disease Control, Atlanta, Ga.; UCSC, Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Rome, Italy; UP, Istituto di Microbiologia, Università di Parma, Parma, Italy). The cultures are listed in numerical order in Table 1. Thirty fungal isolates and the achlorophyllous microorganism were obtained from our culture collection (UCSC) or were kindly furnished by other institutions (CBS [see above]; ISS, Istituto Superiore di Sanità, Rome, Italy; NCMH, M. R. McGinnis, North Carolina Memorial Hospital, Chapel Hill; and J. Frisvad, Food Technology Laboratory, Lyngby, Denmark) (Table 2). Twenty bacterial strains (including the aerobic actinomycete) were isolated from clinical specimens or from soil in our laboratory (Table 2).

A commercially available modified Sabouraud agar (Difco Laboratories, Detroit, Mich.) buffered at pH 4.5 with dibasic anhydrous 0.1 M citric acid–0.2 M potassium phosphate containing 0.003% methylene blue was used for hyphomycetes and bacteria. A different medium, Yeast Morphology Agar (Difco) containing 1% yeast extract (Difco) and 5%

TABLE 1. Recognized killer yeasts tested against procaryotic and eucaryotic microorganisms

Code	Species	Strain[a]
K1	*Hansenula* sp.	Stumm 1034
K2	*Pichia* sp.	Stumm 1035
K3	*Hansenula anomala*	UM 3
K4	*Hansenula anomala*	CBS 5759
K5	*Hansenula anomala*	Ahearn UM866
K6	*Hansenula californica*	Ahearn WC40
K7	*Hansenula canadensis*	Ahearn WC41
K8	*Hansenula dimennae*	Ahearn WC44
K9	*Hansenula mrakii*	Ahearn WC51
K10	*Pichia kluyveri*	Stumm 1002
K11	*Hansenula anomala*	UT 12
K12	*Hansenula bimundalis*	Ahearn WC38
K13	*Hansenula fabianii*	CBS 5640
K14	*Hansenula petersonii*	Ahearn WC53
K15	*Pichia guilliermondii*	UT 19
K16	*Saccharomyces cerevisiae*	CDC B2210
K17	*Hansenula bimundalis*	CBS 5642
K18	*Hansenula fabianii*	Ahearn WC45
K19	*Hansenula holstii*	CBS 4140
K20	*Hansenula subpelliculosa*	CBS 5767
K21	*Pichia ohmeri*	CBS 5367
K22	*Candida guilliermondii*	UCSC 0
K23	*Candida maltosa*	UCSC 0
K24	*Pichia spartinae*	UCSC 0
K25	*Hansenula nonfermentans*	UM 200
K26	*Pichia carsonii*	CBS 810
K27	*Pichia farinosa*	CBS 185
K28	*Pichia guilliermondii*	CBS 2031
K29	*Candida pseudotropicalis*	UP 241
K30	*Candida pseudotropicalis*	UP 254
K31	*Candida pseudotropicalis*	UP 330
K32	*Pichia kluyveri*	UP 5F
K33	*Pichia kluyveri*	UP 6F
K34	*Pichia membranaefaciens*	UP 10F
K35	*Pichia kluyveri*	UP 11F
K36	*Hansenula anomala*	UP 25F

[a] Designations are described in the text.

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TABLE 2. Evaluation of the yeast killer phenomenon in microorganisms

Organism tested[a]	Killer yeasts with inhibitory activity[b]
Staphylococcus aureus	
UCSC 1	2–9, 23, 35
UCSC 2	1–11, 13, 14, 18, 20, 22, 23, 25, 29–33, 35, 36
Citrobacter freundii	
UCSC 0	1–11, 13–16, 18, 20, 24, 27–36
Enterobacter cloacae	
UCSC 1	1–11, 13–16, 18, 20, 29–36
Escherichia coli	
UCSC 1	1–9, 11, 13–16, 18, 29–31, 36
UCSC 3	1–9, 11, 13–15, 18, 20, 29–31, 36
UCSC 4	1–9, 11, 13–16, 18, 20, 21, 23, 24, 29–36
UCSC 5	1–11, 13–16, 18, 20, 29–31, 33–36
Klebsiella oxytoca	
UCSC 1	3–9, 11, 13, 14, 16, 18, 31, 34, 36
UCSC 2	1–11, 13, 14, 16, 18–21, 29–32, 34, 36
UCSC 3	1–11, 13, 14, 16, 18–20, 25, 29–36
UCSC 5	1–11, 13, 14, 16, 18, 20, 29–36
Klebsiella pneumoniae	
UCSC 43	1, 3–8, 10, 11, 14, 15, 34, 36
UCSC 54	1–11, 13–15, 18, 20, 27, 29–31, 34, 36
UCSC 56	1–11, 13, 14, 18, 20, 34, 36
UCSC 60	1–11, 13–15, 20, 33, 34, 36
Pseudomonas aeruginosa	
UCSC 1	1–11, 13, 14, 16, 18, 29–31, 36
UCSC 3	1–11, 13–16, 18, 20, 27–36
Serratia marcescens	
UCSC 1	1–11, 13–15, 18, 29–31, 33, 34, 36
Nocardia asteroides	
UCSC 0	1, 2, 9
Malassezia furfur	
ISS F1	3–5, 8, 11, 13, 14, 16, 18, 20, 36
ISS F8	8, 11, 13, 14, 16, 18, 20, 36
Malassezia pachydermatis	
ISS P2	1–6, 20, 29–31, 36
ISS P5	1–6, 8, 9, 11, 21, 29–31, 36
Aspergillus flavus	
UCSC 0	1, 3–24, 26–29, 32–36
UCSC 2	4–24, 28, 30, 33–36
Aspergillus fumigatus	
UCSC 4	3–11, 13, 20, 21, 27, 33–36
Aspergillus nidulans	
UCSC 0	1–12, 17–20, 22, 30, 32–36
Aspergillus niger	
UCSC 0	9–11, 20, 21, 29–31, 33–36
UCSC 1	1–9, 11–13, 19, 21, 22, 29–31, 36
UCSC 2	1, 2, 5–9, 11–16, 21, 26–29, 32–36
Aspergillus parasiticus	
CBS 103.13	2–8
Aureobasidium pullulans	
UCSC 1	1–9, 11, 13, 20, 36
Cunninghamella elegans	
CBS 161-28	1–9, 20, 21, 23, 29–31, 36
Curvularia sp.	
NCMH 2007	3–11, 17–24, 26, 27, 29–32, 34–36
Exophiala jeanselmei	
UCSC 2	1–25, 29–31, 33–36
Fonsecaea pedrosoi	
UCSC 2	1–14, 16–26, 29–36
Penicillium camembertii	
Frisvad BB	5–9, 11, 12, 19, 21, 27, 29–31, 35, 36
Frisvad PD	5–9, 27, 29–31, 34–36
Penicillium melanochlorum	
Frisvad FTLS 193	1, 2, 9–29, 32–36

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TABLE 2—*Continued*

Organism tested[a]	Killer yeasts with inhibitory activity[b]
Penicillium notatum UCSC 2	1–36
Penicillium palitans Frisvad AMAS 4	1, 4–29, 31–36
Phaeoannellomyces werneckii UCSC 1	1–9, 11–20, 22–24, 29–31
UCSC 2	1, 2, 9, 11, 30–32, 36
Phialophora verrucosa UCSC 0	1–8, 10, 11, 17, 24, 26–28, 33–36
Pseudallescheria boydii UCSC 1	1–8, 11, 13, 17, 20, 34, 36
Rhizopus microsporus var. *microsporus* CBS 699.68	1–8, 21
Scopulariopsis brevicaulis UCSC 1	1–11, 20, 21, 23, 25, 26, 28–32, 36
Sporothrix schenckii UCSC 0	1–9, 21, 29–31, 36
Xylohypha bantiana UCSC 0	1, 2, 4–8, 29–31
Prototheca stagnora UCSC 0	1–9, 11–16, 20, 22–24, 27, 28, 36

[a] Strain designations are described in the text.
[b] Active killer (K) yeasts are numbered according to the codes given in Table 1. Inactive killer yeasts are not listed.

Tween 40 (E. Merck AG, Darmstadt, Federal Republic of Germany) and buffered at pH 4.5, was adopted for culturing the isolates of *Malassezia furfur* and *Malassezia pachydermatis* according to their individual nutritional requirements.

Twenty-four-hour bacterial cultures grown on McConkey agar (Difco) or blood agar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C were suspended in physiological saline. The *Malassezia* spp. isolates were grown on Yeast Morphology Agar for 3 days at 28°C to obtain a heavy suspension in distilled sterile water, as were the hyphomycetes, which were cultured for 7 days on Sabouraud dextrose agar (Difco).

The suspensions were adjusted to an optical density of approximately 25% at 530 nm; 1 ml 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 isolate to be tested. The plates were incubated at 25°C.

Once incubated, the plates were observed daily until there was evidence of growth of the sensitive strain (24 to 72 h for bacteria and longer for the other microorganisms). The killer effect was considered positive either for bacteria (Fig. 1) or for fungi (Fig. 2) when a clear zone of inhibition surrounded the killer colony.

Yeast killer toxins appeared to be inhibitory for a wide variety of procaryotic and eucaryotic organisms. The highest activity was observed in the *Hansenula* species, as expected. Each bacterial or fungal isolate (Table 2) which grew

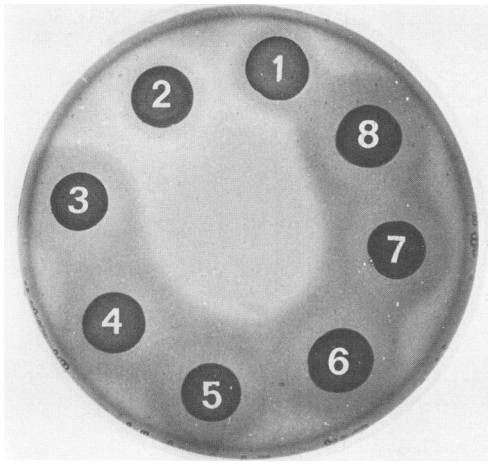

FIG. 1. Inhibitory effect of the first eight killer yeasts on *Citrobacter freundii* UCSC 0. Killer yeasts 1 and 2 displayed a weaker activity during the period of observation.

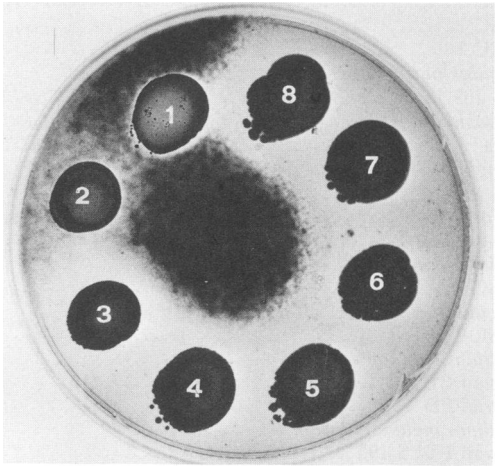

FIG. 2. Inhibitory effect of the first eight killer yeasts on *Pseudallescheria boydii* UCSC 1. Killer yeasts 1 and 2 displayed a weaker activity during the period of observation.

under the experimental conditions was found to be sensitive to at least one killer yeast. The inhibitory effect of the various killer yeasts was expressed differently against various species and strains within the same species.

Until now, there have been no reports of the occurrence of sensitivity to yeast killer toxins among bacteria and eucaryotic microorganisms other than yeasts. The toxic substance produced by some strains of *C. albicans* that inhibits *Neisseria gonorrhoeae* (2) is not a killer toxin, because it does not affect other yeast isolates.

Although the inhibition observed in this work might not necessarily be due to killer toxins but to a variety of different metabolic products, the fact that recognized killer yeasts have displayed their toxic activity in the classic procedure for detecting the killer phenomenon led us to extend that definition to unrelated microorganisms.

We report here, for the first time, the occurrence of yeast killer activity against bacteria, aerobic actinomycetes, hyphomycetes, achlorophyllous microorganisms, and lipophilic yeasts.

By analogy with a number of gram-negative and gram-positive bacteria, which produce bacteriocins (3, 10), and the classic killer phenomenon among yeasts, the yeast killer activity in bacteria and hyphomycetes is expressed differently against different strains within the same species. This finding implies the possible extension of the killer system (7) to bacteria and hyphomycetes for differentiating strains of the same species for epidemiologic purposes.

Finally, the occurrence of the killer phenomenon among yeasts, bacteria, aerobic actinomycetes, hyphomycetes, and achlorophyllous microorganisms, if confirmed with purified killer toxins, implies a unique form of bioaction. Such a study is under way in our institute.

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