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High Incidence of Sensitivity to Yeast Killer Toxins Among *Candida* and *Torulopsis* Isolates of Human Origin

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Among yeast strains of human origin belonging to the genera *Candida*, *Cryptococcus*, *Torulopsis*, and *Rhodotorula* which were examined for killer and sensitive characteristics with killer and sensitive strains of *Cryptococcus*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, and *Torulopsis* as screening organisms, a high incidence of sensitivity to killer toxins was observed within the genera *Candida* and *Torulopsis*. Of 142 strains tested, 116 strains distributed over all *Candida* and *Torulopsis* species examined were sensitive to one or more killers. Several new intergeneric killer-sensitive relationships are described. Furthermore, killing activity was exhibited by six strains of *Candida* (*C. krusei*, *C. guilliermondii*) and three strains of *Torulopsis* (*T. glabrata*).

Killer-sensitive relationships are observed between yeast strains of various genera and species (10, 13) and both inter- and intrageneric interactions are established. (2, 10, 13; E. A. Bevan and M. Makower, Proc. Int. Cong. Genet. XI 1: 203, 1963). Killer toxin-producing strains appear to be immune to the action of their own toxin. However, several reports (2, 11, 13, 15, 17) show that killer strains may be sensitive to the killing action of toxins produced by other strains, and even interactions between two different killer yeasts are observed in which both toxins bypass each others immunity system (2, 3, 11, 17). The toxins are characterized as (glyco-)proteins of low molecular weight and are supposed to act on the yeast plasma membrane by changing its permeability properties (1, 12).

Among the asporogenous yeasts, sensitive and killer properties were previously reported within the genera *Candida* (10, 11, 13, 17), *Cryptococcus* (13), and *Torulopsis* (2, 10, 13, 17). Recently, Kandel and Stern (6) surveyed the frequency of naturally occurring killer and sensitive strains in potentially pathogenic yeast strains from the genera *Candida*, *Cryptococcus*, *Torulopsis*, and *Trichosporon*. In this study a number of hitherto unreported relationships were identified (e.g., killing of *Cryptococcus* and *Candida* species by *Saccharomyces* and *Torulopsis*). Furthermore, they found that killer and sensitive characteristics were exhibited by 3 and 11% of the 236 strains tested, respectively. It is noteworthy that none of the 120 strains of *Candida albicans* tested in this investigation could be identified as killer or sensitive.

The present paper describes results concerning a screening for killer-sensitive properties among isolates of the opportunistic human pathogens of the genera *Candida* and *Torulopsis*. This study was undertaken because the pathogenic yeasts were left out of consideration in a former study (13). Moreover, we had at our disposal several strains of known killer status (killer or sensitive) of the genera *Cryptococcus*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces* and *Torulopsis*, which could be used as appropriate screening organisms in this survey.

MATERIALS AND METHODS

Microorganisms. The yeast strains under investigation were isolated from various clinical sources (e.g., feces, sputum, urine, blood, mouth, vagina, cervix, throat) in the Department of Medical Microbiology. Determination of the strains was performed by the method of English (4). Additional isolates were obtained from J. Beertema, Department of Bacteriology, Centraal Ziekenhuis, Alkmaar (9 *Candida albicans*, 3 *Candida tropicalis*, 1 *Candida parapsilosis*, and 6 *Torulopsis glabrata*), C.F.A. Heyen, Department of Bacteriology, St. Elisabeth Ziekenhuis, Tilburg (15 *C. albicans*), and M. Rozenberg-Arska, Department of Clinical Bacteriology, Academisch Ziekenhuis, Utrecht (24 *C. albicans* and 1 *C. tropicalis*). Eight strains from the Centraalbureau voor Schimmelcultures (*C. albicans* CBS 562; *Candida guilliermondii* CBS 566; *Candida krusei* CBS 573; *C. parapsilosis* CBS 604; *Candida pseudotropicalis* CBS 607; *Candida stellatoidea* CBS 1905; *C. tropicalis* CBS 94; *T. glabrata* CBS 138) and *Cryptococcus neoformans* strain 15 (7) were also included in the screening. Killer (k) and sensitive (s) *Saccharomyces* strains (*S. cere-*

visiae A 8209 B [k/s] and SCF 1717 [s]) were kindly provided by G.R. Fink; killer and sensitive strains from the genera *Cryptococcus* (*Cryptococcus laurentii* 1026 [k]; *Cryptococcus albidus* 1038 [s]), *Hansenula* (*Hansenula* sp. 1034 [k]), *Kluyveromyces* (*Kluyveromyces* sp. 1024 [k]), *Pichia* (*Pichia kluyveri* 1002 [k]; *Pichia* sp. 1035 [k]) and *Torulopsis* (*Torulopsis* sp. 1027 [k]) were isolated during an earlier study (13). The strains were subcultured each fortnight on YEPD agar slants (1% yeast extract, 2% peptone, 2% glucose, 2% agar) and stored at 4°C.

Detection of killer-sensitive relationships. Clinical isolates, CBS strains, and *C. neoformans* strain 15 were tested for killer or sensitive properties or both in five separate series (of 11, 21, 10, 20, and 21 strains, respectively) in the following way. Organisms to be tested were grown for 18 h in 10 ml of 0.1 M citric acid-K_2HPO_4-buffered YEPD medium, pH 4.5, at 25°C in a New Brunswick Gyrotory shaker at 110 rpm. A 100-fold dilution of the culture in sterile YEPD medium was prepared, and 1 ml of this dilution was mixed in a petri disk with YEPD agar medium buffered at pH 4.5 (0.1 M citric acid-K_2HPO_4) and containing 0.003% methylene blue. In every series all killer and sensitive indicator strains of the genera *Cryptococcus*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, and *Torulopsis* were included, and seeded agar plates of these strains (in the last three series of the sensitive indicator strains only) were prepared as described above. All isolates of the series under investigation and the indicator strains were inoculated onto the seeded agar plates. In addition 50 μl of 100-fold-concentrated preparation of *P. kluyveri* 1002 toxin (8) was put in a well (7 mm) in these plates, which were then incubated at 25°C for 24 to 48 h. If an inoculated strain (or the well) was surrounded by a region of bluish-colored cells or by a clear zone of inhibition bounded by colored cells, it was designated as a killer strain, and the seeded strain was designated as a sensitive one. Every isolate, which was identified as a killer within a series, was included in all subsequent series. The isolates obtained from the other hospitals were tested for sensitivity with the indicator killer strains and for toxin production with the indicator sensitive strains.

RESULTS

Sensitivity to killer toxins of yeast strains from different genera. Seventy-four yeast isolates were tested for sensitivity to killer toxins produced by strains of *Cryptococcus*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, and *Torulopsis* at pH 4.5 by the seeded agar technique (14) with methylene blue as dye for killed cells. The pH value of testing was chosen since most killer strains produce toxins, which are active at this pH (8, 10, 14, 16). Of the 74 strains tested, 59 exhibited sensitivity to one or more of the killer strains used, and sensitivity was distributed with high frequency among all *Candida* and *Torulopsis* species examined. Detailed results of the screening are presented in Table 1, which shows all killer-sensitive relationships observed between the reference killer strains and isolates and the intensity of these interactions. In this table, isolates of the same genus and species are grouped according to similar patterns of sensitive properties. Several hitherto unreported relationships were established: (i) with the exception of one strain of *C. albicans*, all isolates of the species examined (*C. albicans*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *T. glabrata*) which were identified as being sensitive were killed by one of the two *Pichia* killers; (ii) *Hansenula* sp. 1034 killed the larger part of the isolates of all species tested; (iii) *Kluyveromyces* sp. 1024 killed several strains of *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *T. glabrata*; and finally (iv) *S. cerevisiae* A8209B killed one strain of both *C. parapsilosis* and *C. tropicalis* and a number of *C. krusei* and *T. glabrata* strains. However, a few strains were identified as sensitive only with a 100-fold-concentrated preparation of the toxin produced by *P. kluyveri* 1002, whereas these strains were not killed by inocula of this killer. Similar observations were reported by Kandel and Stern (6), who demonstrated sensitivity of several strains only with concentrated toxins. This suggests that an even higher frequency of sensitivity may be found when the isolates should be tested with high-titer solutions of the toxins of the killer strains.

No interactions were observed between the killer strains *Kluyveromyces* sp. 1024 and *S. cerevisiae* A 8209B and isolates of *C. albicans* and *C. guilliermondii*. Furthermore, none of the tested isolates was found to be sensitive to representative members (*C. laurentii* 1026 and *Torulopsis* sp. 1027) of the second killer group described by Stumm et al. (13). The one strain of *Rhodotorula* under investigation did not show sensitivity to any of the killers used. The results presented in Table 1 show that *Hansenula* sp. 1034 and *Pichia* sp. 1035 have a much broader spectrum of action than the other killer strains used in this study, which confirms the result of an earlier report (13).

Regarding the frequency of sensitivity, the aforementioned results differ significantly from other studies (6, 13). To ensure that these results do not only represent a local situation but have a more general validity, 59 isolates of *Candida* and *Torulopsis* species from other hospitals (see Materials and Methods) were tested. The results of this additional screening (data not shown) confirm those presented in Table 1 with respect to both incidence (51 strains were sensitive) and patterns of sensitivity.

In addition to the 133 clinical isolates of *Candida*, *Torulopsis*, and *Rhodotorula*, 8 strains of the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and a strain of *C. neoformans* (7) were included in the test procedure. The interactions of these strains with the seven reference killer strains are shown in Table 2. Six of the nine strains examined were sensitive to

TABLE 1. *Killer-sensitive interactions among killer yeasts of different genera and clinical isolates of the genera Candida, Torulopsis, and Rhodotorula*

Seeded strains	No. of isolates tested	Group[a]	Degree of killing for:[b]				
			Hansenula sp. 1034	*Kluyveromyces* sp. 1024	*P. kluyveri* 1002	*Pichia* sp. 1035	*S. cerevisiae* A8209B
C. albicans	38	A (22)	1–2	—	—	1–2	—
		B (2)	2	—	1–2	2	—
		C (2)	—	—	—	2	—
		D (1)	2	—	—	—	—
C. guilliermondii	3	A (1)	2	—	—	2	—
C. krusei	5	A (3)	—	—	1[c]	-	—
		B (2)	(1)	1–2	1–2	2	1–2
C. parapsilosis	9	A (4)	2	2	—	2	—
		B (2)	2	2	1–2	2	—
		C (2)	2	—	—	2	—
		D (1)	2	2	2	2	2
C. tropicalis	7	A (3)	2	—	—	2	—
		B (2)	2	1–2	—	2	—
		C (1)	2	2	2	2	2
T. glabrata	11	A (3)	1	—	1–2	(1)	2
		B (2)	1–2	2	2	1–2	2
		C (2)	—	1–2	2	(1)	2
		D (2)	—	—	2	(1)	1
		E (1)	2	2	—	2	—
		F (1)	—	—	1[c]	—	—
Rhodotorula sp.	1		—	—	—	—	—

[a] Isolates are grouped according to similar sensitive properties. The number of isolates is given within parentheses.

[b] The degree of killing is indicated as follows: — = no killing; (1) = no or weak killing within a group; 1 = weak killing (region of colored colonies <2 mm); 2 = strong killing (region of colored colonies, sometimes together with a clear zone of inhibition, ≥2 mm).

[c] The interactions were only identified with a 100-fold-concentrated preparation of the toxin.

TABLE 2. *Killer-sensitive interactions among killer yeasts of different genera and CBS-strains of the genera Candida and Torulopsis and C. neoformans strain 15*

Seeded strains[a]	Degree of killing for:[b]						
	Hansenula sp. 1034	*Kluyveromyces* sp. 1024	*P. kluyveri* 1002	*Pichia* sp. 1035	*S. cerevisiae* A8209B	*C. laurentii* 1026	*Torulopsis* sp. 1027
C. albicans CBS 562	2	—	2	2	—	—	—
C. pseudotropicalis CBS 607	—	2	2	—	—	—	—
C. stellatoidea CBS 1905	2	—	2	2	—	—	—
C. tropicalis CBS 94	2	—	—	2	—	—	—
T. glabrata CBS 138	2	—	2	2	2	—	—
C. neoformans strain 15	—	—	—	—	—	2	2

[a] *C. guilliermondii* CBS 566, *C. krusei* CBS 573, and *C. parapsilosis* CBS 604 were also tested but did not show sensitivity to any of the killer strains used.

[b] The degree of killing is indicated as in Table 1.

two or more of these killer strains, and sensitivity was established within two new *Candida* species, namely *C. pseudotropicalis* and *C. stellatoidea*. The patterns of sensitivity observed for the CBS strains of *Candida* and *Torulopsis* are in good agreement with those found for the clinical isolates. *C. neoformans* strain 15 was only killed by strains of *Cryptococcus* and *Torulopsis*, which represent a separate group of killer-sensitive strains (13). However, the detection of sensitivity among *C. neoformans* to killer strains of *S. cerevisiae* (6), which probably belong to the other killer-sensitive group observed by Stumm et al. (13), suggest that *Cryptococcus* strains, like *Torulopsis* strains (reference 13 and this paper), may belong to either of both killer-sensitive groups described by these authors.

Killer properties among isolates of the human yeast pathogens *Candida* and *Torulopsis*. In the test procedure, which was described in Materials and Methods, sensitive strains of *Saccharomyces* (*S. cerevisiae* SCF 1717 and A 8209 B) and of *Cryptococcus* (*C. albidus* 1038) were included to detect killing activity among the strains to be tested. Of 142 strains examined, killer properties were observed for 9 strains including all 5 isolates of *C. krusei*, 1 of *C. guilliermondii*, and 3 of *T. glabrata* (including *T. glabrata* CBS 138). Sensitive strain *S. cerevisiae* SCF 1717 was killed by all these killers. From these strains, the isolates of *C. krusei* and a strain of *T. glabrata* did not show further interactions, whereas *C. guilliermondii* killed an isolate of *C. tropicalis*, which was also affected by one of the *T. glabrata* killers. This strain of *T. glabrata* also interacts with one isolate of *C. parapsilosis* and three of *T. glabrata*, including both other killers identified within this species. Finally, *S. cerevisiae* A 8209 B exhibited sensitivity to *T. glabrata* CBS 138.

DISCUSSION

Recently, Kandel and Stern (6) surveyed the killer phenomenon in potentially pathogenic yeasts and found approximately 3 and 11% of the 236 strains tested to show killer or sensitive characteristics, respectively. These authors used *Saccharomyces* and *Torulopsis* strains of known killer status for the examination of both killer activity and sensitivity among the strains to be tested. The present study describes the results of a similar screening with a number of strong killer strains belonging to several other yeast genera, namely *Cryptococcus*, *Hansenula*, *Kluyveromyces*, and *Pichia* as screening organisms besides killer and sensitive strains of *S. cerevisiae* and *Torulopsis*. With these tester strains, results were obtained which differ markedly from those of Kandel and Stern (6); especially, the frequency of occurrence of sensitive properties among the isolates tested was found to be several times higher in our screening. Of 142 strains tested, sensitivity to one or more killers was observed for 116 strains distributed over all *Candida* and *Torulopsis* species examined. For *C. albicans* (70 of the 87 strains were sensitive) and *T. glabrata* (all 17 strains tested were sensitive), which are the two most common pathogenic yeasts (5), the frequencies of sensitivity were established to be 79 ± 9% and 100 − 20%, respectively. However, with the killer strain of *S. cerevisiae* (A 8209 B) only 20 strains were detected as sensitive, and according to the results of Kandel and Stern (6) no sensitivity was found among isolates of *C. albicans* with this tester strain. In contrast to these authors, who could not detect killer properties within the genus *Candida*, killing activity was exhibited by six strains of *Candida* (five *C. krusei*; one *C. guilliermondii*). These data clearly show the importance of the choice of killer and sensitive screening strains and can give an explanation for the apparent underestimation of the frequency of sensitive properties among the pathogenic yeasts as found by Kandel and Stern (6). It should be interesting to investigate *Candida* and *Torulopsis* strains from other than human origin to see whether a similar high incidence of sensitive properties is found among these strains. However, the factors underlying the phenomenon of high frequency of sensitivity, which was not yet observed in other yeast genera, remain unclear.

A possible role of yeast killer toxins as antifungal drugs in the treatment of infections due to the human yeast pathogens, as suggested by the high incidence of sensitive properties among these strains, would be limited because of their lability at neutral pH and at elevated temperature.

LITERATURE CITED

1. **Bussey, H., and D. Sherman.** 1973. Yeast killer factor: ATP leakage and coordinate inhibition of macromolecular synthesis in sensitive cells. Biochim. Biophys. Acta **298**:868–875.
2. **Bussey, H., and N. Skipper.** 1975. Membrane-mediated killing of *Saccharomyces cerevisiae* by glycoproteins from *Torulopsis glabrata*. J. Bacteriol. **124**:476–483.
3. **Bussey, H., and N. Skipper.** 1976. Killing of *Torulopsis glabrata* by *Saccharomyces cerevisiae* killer factor. Antimicrob. Agents Chemother. **9**:352–354.
4. **English, M. P.** 1974. Identifying yeasts. Med. Lab. Technol. **31**:327–333.
5. **Gentles, J. C., and C. J. La Touche.** 1969. Yeasts as human and animal pathogens, p. 107–182. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, vol. 1. Academic Press Inc., London.
6. **Kandel, J. S., and T. A. Stern.** 1979. Killer phenomenon

in pathogenic yeast. Antimicrob. Agents Chemother. **15**:568–571.

7. **Maccani, J. E.** 1977. Detection of cryptococcal polysaccharide using counterimmunoelectrophoresis. Am. J. Clin. Pathol. **68**:39–43.
8. **Middlebeek, E. J., J. M. H. Hermans, and C. Stumm.** 1979. Production, purification and properties of a *Pichia kluyveri* killer toxin. Antonie van Leeuwenhoek J. Microbiol. Serol. **45**:437–450.
9. **Palfree, R. G. E., and H. Bussey.** 1979. Yeast killer toxin: purification and characterization of the protein toxin from *Saccharomyces cerevisiae*. Eur. J. Biochem. **93**:487–493.
10. **Philliskirk, G., and T. W. Young.** 1975. The occurrence of killer character in yeasts of various genera. Antonie van Leeuwenhoek J. Microbiol. Serol. **41**:147–151.
11. **Rogers, D., and E. A. Bevan.** 1978. Group classification of killer yeasts based on cross-reactions between strains of different species and origin. J. Gen. Microbiol. **105**: 199–202.
12. **Skipper, N., and H. Bussey.** 1977. Mode of action of yeast toxins: energy requirement for *Saccharomyces cerevisiae* killer toxin. J. Bacteriol. **129**:668–677.
13. **Stumm, C., J. M. H. Hermans, E. J. Middelbeek, A. F. Croes, and G. J. M. L. de Vries.** 1977. Killer-sensitive relationships in yeasts from natural habitats. Antonie van Leeuwenhoek J. Microbiol. Serol. **43**:125–128.
14. **Woods, D. R., and E. A. Bevan.** 1968. Studies on the nature of the killer factor produced by *Saccharomyces cerevisiae*. J. Gen. Microbiol. **51**:115–126.
15. **Woods, D. R., I. W. Ross, and D. A. Hendry.** 1974. A new killer factor produced by a killer/sensitive yeast strain. J. Gen. Microbiol. **81**:285–289.
16. **Young, T. W., and G. Philliskirk.** 1977. The production of a yeast killer factor in the chemostat and the effects of killer yeasts in mixed continuous culture with a sensitive strain. J. Appl. Bacteriol. **43**:425–436.
17. **Young, T. W., and M. Yagiu.** 1978. A comparison of the killer character in different yeasts and its classification. Antonie van Leeuwenhoek J. Microbiol. Serol. **44**:59–77.