

## Killer Phenomenon in Pathogenic Yeast

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Potentially pathogenic yeast strains from four genera, *Candida*, *Cryptococcus*, *Torulopsis*, and *Trichosporon*, were examined for killer activity and sensitivity using *Saccharomyces* and *Torulopsis* strains of known killer status. Tests were performed by using a streak method and by concentration of culture fluid by lyophilization. Of 236 strains examined, killers were found with low prevalence among *Torulopsis* and *Cryptococcus* strains; *Candida* and *Trichosporon* isolates showed no killing activity. Different specificities of killing activity were observed among strains of a single species. Sensitive strains were found with varying frequencies in all genera but *Trichosporon*.

Strains of *Saccharomyces cerevisiae* which produce an extracellular protein toxin capable of killing closely related sensitive strains have been described (12). The ability to produce this killer toxin is related to the presence of double-stranded ribonucleic acid containing virus-like particles in the yeast cytoplasm (11). A similar phenomenon has been described in the yeastlike corn smut *Ustilago maydis* (6). The killer phenomenon in fungi appears to be fundamentally similar to the bacteriocin phenomenon in prokaryotes. Genetic determinants for both products are of a cytoplasmic nature; the products are protein and the effects on their specific target cells appear to be similar (1, 3).

Few reports have addressed the question of frequency of occurrence and range of specificity of these toxins. Philliskirk and Young (7), in screening 28 genera from the National Collection of Yeast Cultures in Britain, found killers in 7 genera. Strains sensitive to a given killer were not necessarily from the same genus. In addition, killers from one genus could kill killers from other genera (13). Stumm et al. (8) examined 157 isolates from various natural habitats and identified one additional genus with killer activity. In addition, they reported killer-sensitive relationships in three genera not previously examined. In 1976, Bussey and Skipper (2), using a killer *Saccharomyces* strain to screen for sensitive yeasts, reported the detection of a sensitive strain of *Torulopsis glabrata*. This was the first description of the killing of a human pathogenic yeast by the killer products from a nonpathogen. Here we report the results of screening a variety of yeast isolates from clinical, laboratory, and autopsy sources to determine the frequency of naturally occurring killer and sensitive strains in potentially pathogenic yeasts.

### MATERIALS AND METHODS

**Organisms.** The yeast strains were obtained from various hospitals, educational institutions, and the American Type Culture Collection (ATCC). These consisted of 154 *Candida* strains (20 species), 41 *Cryptococcus* strains (7 species), 39 *Torulopsis* strains (10 species), and 2 *Trichosporon* strains (2 species). Eleven *Saccharomyces* strains of known killer status were obtained from R. Wickner, National Institutes of Health, Bethesda, Md., T. Young, University of Birmingham, England, and J. Adler, California Polytechnic University, Pomona. Stock cultures were maintained on YPD agar slants (1.0% yeast extract, 2.0% peptone, 2.0% dextrose, and 2.0% agar) at room temperature and subcultured every 2 weeks.

**Killing by living cells.** *S. cerevisiae* 5 × 47, *S. cerevisiae* F, and *T. glabrata* T1, verified sensitive strains, were each grown in 125-ml flasks with 50 ml of buffered YPD broth (0.5% yeast extract, 1.0% peptone, and 2.0% dextrose in 0.05 M potassium citrate buffer) for 24 h at room temperature on a Brunswick gyratory shaker. Cultures were grown at both pH 4.2 and 4.7.

After incubation, the cells grown at pH 4.2 were added to pH 4.2 YPD agar, the cells grown at pH 4.7 were added to pH 4.7 agar at a 1:150 dilution, and seeded petri plates were prepared.

The seeded plates were streaked with thick smears of 48-h cultures being tested for killer activity. Plates were incubated at room temperature and observed for inhibition of growth of the seed culture surrounding the streak of the strain being tested.

**Killing by isolated toxin.** Cultures to be tested for killer toxin production were grown in 125-ml flasks, each containing 50 ml of unbuffered YPD broth. Incubation was for 48 to 72 h at room temperature with shaking. After incubation, cultures were centrifuged 12,000 × g for 10 min. The resulting supernatant was filtered through sterile membrane filters (Millipore Corp., type HA, 0.45 μm). Filtrates were quick-frozen and lyophilized to dryness. The resulting lyophilizates were dissolved in sterile distilled water and divided in half. One half was adjusted to pH 4.2, and the other was adjusted to pH 4.7 with potassium citrate buffer

to a final concentration of 0.05 M. The resulting killer toxin (if present) had been concentrated 25 $\times$ .

Seeded plates were prepared as described. Wells with a diameter of 4 mm were cut into the agar plates. The wells were filled with 75  $\mu$ l of resuspended (25 $\times$ ) culture filtrate and incubated at room temperature, and plates were observed for inhibition of growth surrounding the well.

**Sensitivity to killer.** Organisms to be tested for sensitivity were grown in unbuffered YPD broth for 24 h at room temperature with shaking, and seeded YPD agar plates at pH 4.2 and 4.7 were prepared. A well was punched into each plate and filled with concentration (25 $\times$ ) killer toxin derived from either *S. cerevisiae* SK at pH 4.7 (9) or *T. glabrata* 15126 at pH 4.2 (2). On the remaining sections of agar, 48-h cultures of killers *S. cerevisiae* SK and S-20 (10) were streaked on the pH 4.7 plates, and *T. glabrata* 15126 was streaked on the pH 4.2 plates. Plates were incubated at room temperature, and zones of clearing immediately surrounding the wells and/or streaks indicated sensitivity to the killer toxin.

## RESULTS

**Assays for killer activity.** A total of 236 yeast strains were assayed for killer activity using sensitive strains *S. cerevisiae* 5  $\times$  47 and *T. glabrata* T-1. Strain T-1 was identified as a sensitive strain after preliminary testing with a known *Torulopsis* killer. By using known killers, strain 5  $\times$  47 was shown to be a good indicator of sensitivity at pH 4.7, and T-1 was shown to be a good indicator strain at pH 4.2. In some tests an additional strain, *S. cerevisiae* F, a strain also showing high sensitivity at pH 4.2, was used.

Of 154 *Candida* strains and 41 *Cryptococcus* strains tested, none showed any killer activity at either pH. Similarly, no activity was observed from either of the two *Trichosporon* species tested. Of 39 strains from the genus *Torulopsis*, 7 showed killer activity against F at pH 4.2. Two strains (15126 and N7) were also able to kill strain T-1 at the same pH, whereas only four of these seven were active against strain 5  $\times$  47 at pH 4.7. The activity at the higher pH was noticeably reduced. *Saccharomyces* killer strains were both shown to be killers at pH 4.2; however, activity was more pronounced at pH 4.7. All seven active strains were of the species *T. glabrata*, two of which (15126 and 388) were previously described as killer by Bussey and Skipper (2) and Philliskirk and Young (7), respectively. The other five were newly determined to be killers.

**Assays for sensitivity to known killers.** Sensitivity testing by the well method (isolated toxin) or streak method (killing by living cells) using known killer *S. cerevisiae* SK showed 26 of the 236 strains to be sensitive. These 26 sensitive strains included 2 *C. parapsilosis*, 2 *C.*

*rugosa*, 1 *C. krusei*, 1 *C. guilliermondii*, 1 *Cryptococcus neoformans*, and 19 *T. glabrata* strains. Five of the strains were sensitive to lyophilized and 25-fold-concentrated SK (25  $\times$  SK) but were not inhibited by streaks of SK.

Testing with known killer *S. cerevisiae* S-20 at pH 4.7 identified an additional sensitive *Torulopsis* strain, which was not sensitive to SK. Conversely, 9 of the 11 strains sensitive to SK were not inhibited by S-20.

Sensitivity testing using known *T. glabrata* killer 15126 showed 15 strains to be sensitive at pH 4.2. Among these were two *C. parapsilosis* strains, two *C. rugosa* strains, 1 *C. guilliermondii* strain, two *Cryptococcus neoformans* strains, seven *T. glabrata*, and one *C. tropicalis* strain. Sensitivity of 6 of the 15 strains was demonstrated by using 25  $\times$  15126 toxin only, since these 6 strains were not inhibited by streaks of 15126. In addition, 5 of 8 *Saccharomyces* sensitive to *Saccharomyces* killer were sensitive to *Torulopsis* killer.

Possible killer-sensitive relationships were examined among 10 *Cryptococcus neoformans* strains, 22 *T. glabrata* strains, and 1 strain each of 6 *Candida* species. Each strain was tested for killer activity toward and sensitivity to the other 37 strains at pH 4.2 by the streak method. Five *T. glabrata* killers, strains 15126, 388, N7, N8, and N9, and a killer strain of *C. valida* isolated by Philliskirk and Young could be compared for specificity of killing (Table 1). The range of killing varied among the strains, suggesting differences in toxin structures and specificity even within the species. Both N7 and 15126 showed wide ranges of killing activity. N8 and N9, isolated from different tissues of a single individual after autopsy, have a more restricted range, whereas 388 killed only strains of *Saccharomyces*. *C. valida* also killed a limited range of organisms, *Saccharomyces*, *Torulopsis*, and a single *Candida* species. Within a species, the number and range of organisms exhibiting sensitivity to each of the killers varied. For example, N8 and N9 killed only two strains of *T. glabrata*, whereas N7 killed six strains.

In addition, two strains of *Torulopsis glabrata* were shown to be sensitive to *Cryptococcus neoformans*. The sensitivity appeared to be an inhibitory rather than a killing phenomenon since, upon prolonged incubation, growth developed in the zones surrounding the colonies.

The killer-sensitive relationships among killer strains was determined (Table 2). Strains could be classified according to the spectrum of their activity. *T. glabrata* killers N8, N9, and 388 showed no activity against killers from the same genus, but they were active against *Saccharo-*

TABLE 1. *Killer specificities*

Strain	Sensitivity to killer							
	15126 <sup>a</sup>	388 <sup>a</sup>	N7 <sup>a</sup>	N8 <sup>a</sup>	N9 <sup>a</sup>	SK <sup>b</sup>	S-20 <sup>b</sup>	327 <sup>c</sup>
<i>T. glabrata</i>	+	-	+	+	+	+	+	+
<i>S. cerevisiae</i>	+	+	+	+	+	+	+	-
<i>Cryptococcus neoformans</i>	+	-	+	-	-	+	+	-
<i>Candida parapsilosis</i>	+	-	+	-	-	+	-	+
<i>C. guilliermondii</i>	+	-	+	-	-	+	-	-
<i>C. rugosa</i>	+	-	+	-	-	+	+	-
<i>C. krusei</i>	-	-	-	-	-	+	-	-
<i>C. tropicalis</i>	+	-	+	-	-	-	-	-
<i>C. valida</i>	-	-	+	-	-	-	-	-

<sup>a</sup> *T. glabrata*, assay conducted at pH 4.2.

<sup>b</sup> *S. cerevisiae*, assay conducted at pH 4.7; SK isolated and described by Vodkin et al. (8); S-20 (*α-his1*) obtained from R. Wickner (11).

<sup>c</sup> *C. valida*, assay conducted at pH 4.2.

TABLE 2. *Killer-susceptible relationships among killers*

Strain tested	Strain seeded									
	N6	N7	N8	N9	N26	388	15126	SK	S-20	327
<i>T. glabrata</i>										
N6	-	-	+	+	-	+	-	+	+	+
N7	-	-	+	+	-	+	-	+	+	+
N8	-	-	-	-	-	-	-	+	+	+
N9	-	-	-	-	-	-	-	+	+	+
N20	-	-	+	+	-	+	-	+	+	+
388	-	-	-	-	-	-	-	+	-	-
15126	-	-	+	+	-	+	-	+	+	-
<i>S. cerevisiae</i>										
SK	+	+	-	-	+	+	+	-	-	-
S-20	+	+	-	-	+	+	+	-	-	-
<i>C. valida</i>										
327	+	+	-	-	+	+	=	-	-	-

*myces* killers. Strain 388 showed weak activity against SK and none against S-20. Strains N6, N7, and N26 showed identical patterns. They were able to kill *Torulopsis*, *Saccharomyces*, and *Candida* killers. Strain 15126 killed *Torulopsis* and *Saccharomyces* killers but not *C. valida*.

## DISCUSSION

Of 234 new strains tested, approximately 3% were killers. Since five occurred in the genus *Torulopsis* and three in the genus *Cryptococcus*, it appears that the prevalence of naturally occurring killer strains in pathogenic yeasts is low and may even be limited to one or a few species. Philliskirk and Young (7), in screening 964 yeast strains, found killing activity to be restricted to 7 of 28 genera studied, but killing was found at a prevalence greater than 10% in only 2 genera. Our studies also indicate that approximately 11% of the strains tested exhibit sensitivity to at least one killer. This is in good agreement with the

percentage of sensitive strains detected by Stumm et al. (8).

In this study, several new relationships have been identified; killing of *Cryptococcus* and *Candida* species by *Saccharomyces*; killing of *Torulopsis* and *Candida* species by *Candida*; and killing (inhibition) of *Torulopsis* species by *Cryptococcus*. It is interesting to note that no strains of *C. albicans* (120 were tested) were identified as killers or sensitive, although it has been reported that spheroplasts of *C. albicans* are sensitive to *Saccharomyces* toxin (J. Wilbur-Murphy and J. D. Macmillan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, F30, p. 92). We have also shown that, even within a given genus, the range of activity of a killer may vary and that killers from one genus may kill killers from the same or different genera. The variation in distribution and frequency of observed killers among and within genera and among studies may reflect a variation of sensitive test strains in their sensitivity to different killers under test conditions which may not be optimal for the

organism under study. Alternatively, this may reflect differences in the amount of toxin produced by the various killer strains or differences among the toxins themselves. The latter case is found in *U. maydis* where three distinct specificities of killer toxin, associated with three proteins, have been characterized (4).

The possible functions of killer toxins are numerous, but the frequency with which they are encountered in nature, as shown by these studies and as reported by Koltin and Day (4) for killers in the corn smut *U. maydis*, Philliskirk and Young (6) for a variety of nonpathogenic yeasts, and Stumm et al. (7) for soil isolates, suggests that the ability to produce killers may have little, if any, value as a selective force. Although not very widespread in nature, the killer phenomenon may be more common within specific genera where it may serve a useful role.

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