OCCURRENCE OF KILLER CANDIDA GLABRATA CLINICAL ISOLATES

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ABSTRACT

In this work we characterized the occurrence of killer activity in 64 Candida glabrata clinical isolates under different conditions. We found that only 6.25 % of the clinical isolates tested were positive for killer activity against a Saccharomyces cerevisiae W303 sensitive strain. Sensitivity of killer activity to different values of pH and temperatures was analyzed. We found that the killer activity presented by all isolates was resistant to every pH and temperature tested, although optimal activity was found at a range of pH values from 4 to 7 and at 37°C. We did not observe extrachromosomal genetic elements associated with killer activity in any of the positive C. glabrata isolates. The killer effect was due to a decrease in viability and DNA fragmentation in sensitive yeast.

Key words: Candida glabrata; killer activity; yeast; Saccharomyces

INTRODUCTION

C. glabrata has emerged as an important pathogen in humans and now is the second most common Candida species isolated from bloodstream infections, after Candida albicans (17, 18). This emergence has been attributed to a low susceptibility to azole compounds and to the high rate at which C. glabrata develops resistance to antifungals, requiring the use of alternative antifungal therapy (5, 6, 15, 19, 22). Some yeast strains of Candida and other genera secrete into the extracellular medium proteins or glycoproteins also known as killer toxins with toxic effects on sensitive yeasts (1). It has been reported that the capacity to produce killer proteins can confer advantage over sensitive strains when competing for nutrients available in their host or in the environment (25). Several studies propose such killer proteins as potential novel antimycotic biocontrol agents for fungal pathogens and for treatment of human fungal infections (20, 26). In C. glabrata Muller et al. (16) reports three killer yeast from 182 human clinical isolates, but none of this killer yeast or killer toxin properties were characterized.

Killer proteins were described first in Saccharomyces cerevisiae and soon after in many other yeast such as Candida, Cryptococcus, Debaryomyces, Kluyveromyces, etc. The genetic elements that encode for a killer phenotype are double stranded RNA molecules (dsRNA) encapsulated in virus like particles, linear double stranded DNA plasmids (dsDNA) or nuclear genes (23) and these genetic elements that encode for killer
proteins have been isolated from several yeast genera, and killer phenotypes are classified into at least 11 groups (27, 30). The mechanisms by which killer toxins kill sensitive yeast are: disruption of the membrane function through ion channel formation (9), blockage DNA synthesis, and arrest in the G1 phase of the cell cycle and by caspase-mediated apoptosis (21).

In this work, we analyzed the occurrence of killer phenotype in a collection of 64 clinical isolates of *C. glabrata* and we partially characterized the killer toxin, including pH and temperature sensitivity of the killer activity. We also determined the presence of extra chromosomal genetic elements and killer effect in yeast viability.

**MATERIALS AND METHODS**

**Strains**

Laboratory strains and human clinical isolates used in this work are described in Table 1.

**Table 1. Strains used in this study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent</th>
<th>Genotype</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>W303</td>
<td>MATa ura3-1 leu2-3,112 his3-11,15 trp1 ade2-1 ade3::hisG aMKT&quot;</td>
<td>Lab collection (IPICYT)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> (Containing a 4.5 Kb dsRNAs)</td>
<td>S288C</td>
<td>α MKT+</td>
<td>(28)</td>
</tr>
<tr>
<td><em>Candida glabrata</em> BG14</td>
<td>BG1, clinical isolate</td>
<td>ura3Δ::Tn903 G418R</td>
<td>(2)</td>
</tr>
<tr>
<td><em>Candida glabrata</em> CBS138</td>
<td>Clinical isolate</td>
<td>kill - killer +</td>
<td>Reference strain (ATCC2001)</td>
</tr>
<tr>
<td>Clinical isolates 1 through 64</td>
<td>Clinical isolates</td>
<td></td>
<td>(Lavaniegos-Sobrino et al. 2009)</td>
</tr>
<tr>
<td>MC1 through MC64</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Culture media**

All yeast were grown in standard yeast media YEPD broth containing 10 g/L yeast extract, 20 g/L peptone, 2 % glucose were supplied by Fisher. YPD-MB agar (YPD containing 0.01% methylene blue and 2.0% agar) was used for killer phenotype determination. YPD with 2.0% agar was used for viability assay.

**Toxin crude extracts preparation and determination of killer activity**

Killer cells were inoculated from stock culture and grown in 250 mL Erlenmeyer flasks containing 100 mL of YEPD medium at 30°C and 200 r.p.m. of shaking. Samples of 10 mL of *C. glabrata* culture samples were centrifuged at 3500 rpm for 10 min at 4°C. The supernatant was filtered through sterile 0.22 μm pore size polivinyliden fluoride membrane (Millipore). A volume of ethanol was added to the cell-free supernatant to achieve a final concentration of 70% v/v, incubated 4°C for 1 h and centrifuged at 16,000 g for 40 min. The pellet was dried and resuspended in 1 mL of citrate/phosphate buffer (1 mM sodium citrate/phosphate pH 7). Killer extracts were maintained at -20°C. To evaluate killer activity, 100 μl of killer extracts were impregnated on filters (8 mm diameter) placed on YPD-MB agar plates; each inoculated with 1 x 10^6 cells of the sensitive strain *S. cerevisiae* W303. The plates were incubated at 30°C for 72 h. The killer strains were identified by the presence of a death halo (precipitate of methylene blue) of the sensitive cells surrounding the filter containing the killer extract. The diameter of the inhibition zone around each filter was measured and the area was calculated.

**Effect of the pH and temperature on killer activity**

In order to analyze the effect of pH on killer toxin activity, killer yeast were grown in YEPD medium adjusted to different pH values ranging from 4-9 with citrate-phosphate buffer, then
Killer activity of some *Candida glabrata* clinical isolates

We screened 64 clinical isolates from *C. glabrata* for ability to kill sensitive *S. cerevisiae* strain W303. We found that only four (6.25%) of all the clinical isolates showed killer activity and by comparison, *C. glabrata* CBS-138 (ATCC2001) a previously identified killer yeast (16) produced more killer activity than any of the four clinical isolates identified in this study. Only the killer activity from clinical isolates MC28 was lower than the other isolates (Fig. 1).

**Cell viability assay**

To examine killer toxin activity in liquid media, yeast strains were inoculated in YPD medium and incubated with shaking at 30°C in the presence or absence of the killer extracts for 72 hours. Serial dilutions of each culture were made and aliquots were plated onto YPD agar plates. The colony forming units (CFU) of the yeast was evaluated after 3 days of incubation at 30°C, the results were expressed as % CFU of treated yeast cells compared with untreated control cells.

**Statistical analysis**

Cell viability results are presented as mean ± S.D. of minimal three independent measurements. In addition, Tukey test was employed for further determination of the significance of differences between control and treated cells.

**RESULTS**

![Figure 1](image.png)

**Figure 1.** Killer activity of four *C. glabrata* clinical isolates. Killer yeast (CBS138), killer yeast negative (BG14). n=5.
Effect of pH and temperature on killer activity

As shown in Fig. 2, killer yeast extracts were adjusted to pH values of 4-9 in order to analyze the effect of pH on killer protein stability. We found that killer activity was almost constant over the pH range tested and little difference was observed when *C. glabrata* culture media used for the cultivation of the clinical isolates had their pH adjusted at 4 to 7, but at pH values from 8 to 9 pH killer activity was reduced considerably. Fig. 3 shows the effect of changes in pH on the secretion of killer toxin by *C. glabrata* strains grown at different pH values. Secretion of killer toxin by all producing *C. glabrata* clinical isolates was constant between pH values from 4 to 7, while secretion was markedly decreased when *C. glabrata* clinical isolates were grown at pH values from 8 to 9.

We then determined whether killer activity was affected by the temperature of incubation during the growth assay. As shown in Fig. 4, culture extracts from all *C. glabrata* clinical isolates and from the positive control CBS138, showed stronger killer activity when *S. cerevisiae* sensitive yeast was grown at 37°C and 28°C or 30°C.

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**Figure 2.** Effect of pH on stability of killer activity. Culture filtrates were adjusted to the appropriate pH (4-9) with 0.1 M citrate phosphate buffer and kept at 4°C. Determination of killer activity was assayed. n= 5.

**Figure 3.** Effect of the pH in the *C. glabrata* growth media on the production of killer activity. YPD aliquots were adjusted to the appropriate pH values (4-9) with 0.1 M citrate phosphate buffer. Killer activity was assayed. n=4.

**Figure 4.** Temperature effect on killer activity. The killer activity was assayed using culture filtrates at pH 7.0 and incubated at 25, 28, 30 and 37°C for 72 h. n=4.

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**Plasmid isolation and curing**

It has been previously reported that UV irradiation induces...
removal extrachromosomal genetic elements as dsRNA and dsDNA that encoded killer phenotype (27, 28). This killer plasmids loss by UV is by DNA damage and formation of pyrimidine dimmers that disturbs DNA replication, RNA synthesis and plasmid replication (7). In order to test whether C. glabrata killer yeast contain extrachromosomal genetic elements, total nucleic acids were purified and analyzed by agarose gel electrophoresis. None of the killer clinical isolates of C. glabrata or CBS138 reference strain presented any extrachromosomal band of nucleic acids. Only in our positive control S. cerevisiae S288C strain, an extrachromosomal band of about 4.5 kb was found as described (Fig. 5 A) (28). In order to cure possible extrachromosomal nucleic acids not identified by agarose gel electrophoresis, we irradiated with UV all four killer C. glabrata clinical isolates at 20,000 μJ/cm² (87-98 % of killing). Surviving colonies from all four clinical isolates were tested for killer activity, and all of them, as well as surviving colonies from CBS138 strain retained the killer activity after the exposition to UV (data no shown). These data strongly suggest that the gene is localized in the chromosome.

Killer toxin from C. glabrata induces DNA fragmentation and loss of viability.

Incubation of sensitive yeast S. cerevisiae with killer toxin extracts results in gradual loss of viability after 24, 48 and 72 h treatment. The relative CFU decrease is time dependent in the clinical isolates, but the loss of viability is more pronounced with killer toxin from CBS138 (Fig. 6). To determine if the loss of viability occurs through induction of DNA fragmentation in the sensitive yeast strain of S. cerevisiae (in an apoptotic-like pathway), DNA was extracted from sensitive yeast S. cerevisiae treated with killer toxin for 72 h and resolved in an agarose gel. Figure 7 shows DNA fragmentation (visible as DNA ladders) of varying sizes only in cells treated with killer culture extracts but not in control cells treated with used media from non-killer C. glabrata clinical isolates (Fig. 7 compare lanes 5-9 with lanes 1-3).

Figure 5. Detection of total nucleic acids isolated from C. glabrata clinical isolates by agarose gel electrophoresis. BG14 (killer negative), CBS138 (killer yeast), S. cerevisiae (contain a 4.5 kb dsRNA plasmid used as positive control). gDNA, genomic DNA. dsRNA, double stranded RNA. n=3.

Figure 6. Sensitivity of S. cerevisiae to killer extract from C. glabrata clinical isolates. A, % survival (CFU %) of S. cerevisiae in liquid media incubated with killer extracts, log CFU (%) is the relative number of colony forming units; time 0= 5 x 10⁶ cells. CFU was measured at intervals by diluting and plating on YPD. n= 5.
DISCUSSION

The present study describes the killer phenotype from a collection of *C. glabrata* clinical isolates, our data showed low occurrence (6.25%) of killer yeast from a collection of 64 clinical isolates. This low prevalence of killer pathogenic strains has been previously reported. Kandel and Stern (10), screening 234 strains from *Cryptococcus*, *Torulopsis*, *Candida*, and *Trichosporon* and found killing activity only in 5% *Candida* and 7% in *Cryptococcus*, therefore it appears that occurrence of pathogenic killer yeast is limited.

The majority of killer yeast did not produce killer toxin at pH values higher than pH 5.6 and the optimal pH for killer activity oscillates between 4 to 4.6 (20). We found that CBS138 and our *C. glabrata* clinical isolates produce killer toxin over a wide range of pH and the optimal killer activity was at pH 4 to 8 at 37°C, in agreement with Yokomori et al. (29) where it is shown that SW-55 killer toxin from genus *Candida* had killer activity over a broad pH range and temperature. These properties of *C. glabrata* killer proteins could have potential antimycotic activity against fungal pathogens, although further studies are necessary. In agreement with previous studies where it was shown that the killer protein from *Candida sp* SW-55 is encoded in the chromosome (29), we found that none of the clinical isolates of *C. glabrata* with killer activity have extrachromosomal molecules, suggesting that the genes responsible for this activity are encoded in the genome (23). Furthermore, the fact that killer phenotype was not cured with UV radiation strengthens this notion (Data no shown).

In yeast, apoptotic cell death can be induced by different exogenous and intrinsic stresses like H₂O₂, UV irradiation, acetic acid, cell aging, and high pheromone concentration (4, 8, 11-13, 24). Based on the data presented here, we suggest that killer yeast toxins from *C. glabrata* clinical isolates and CBS138 decrease viability of *S. cerevisiae* sensitive strain, probably through an apoptotic cell death as suggested by DNA fragmentation observed in sensitive yeast. This process has been reported previously with killer toxin k1 and k28 where apoptosis is mediated through yeast caspase Yca1p and the generation of ROS (21). More studies are necessary to analyze the apoptotic cell mechanism induced by *C. glabrata* killer toxins.

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Killer *C. glabrata*
