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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

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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.

Strain	Parent	Genotype	Source or Reference
Saccharomyces cerevisiae	W303	MATa *ura3-1 leu2-3,112 his3-11,15 trp1 ade2-1 ade3::hisG*	Lab collection (IPICYT)
Saccharomyces cerevisiae (Containing a 4.5 Kb dsRNAs)	S288C	αMKT^{+}	(28)
Candida glabrata BG14	BG1, clinical isolate	*ura3*Δ::Tn*903* $G418^{R}$ killer -	(2)
Candida glabrata CBS138	Clinical isolate	killer +	Reference strain (ATCC2001)
clinical isolates 1 through 64 MC1 through MC64	Clinical isolates		(Lavaniegos-Sobrino *et al*. 2009)

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 extracts and were obtained maintained at -20°C. Killer extracts from killer yeast grown at pH 7.0 were adjusted to different pH values ranging from 4 to 9 and then 100 µL aliquots of the samples were added to determine killer activity on YPD-MB agar, previously plated with the sensitive yeast suspension. Three plates of each pH were incubated at 25, 28, 30, 37°C for 72h.

Extraction of total nucleic acids and DNA fragmentation assay

Cells were collected by centrifugation at 3500 rpm for 10 min, resuspended in lysis buffer (50 mM Tris, 10 mM EDTA, 150 mM NaCl, 1% Triton and 1% SDS) plus 500 µL of phenol:chloroform:isoamylic alcohol (25:24:21) followed by incubation at 44 ºC for 30 min. The aqueous phase was recovered and washed twice with 1 volume of cold ethanol and the pellet was resuspended in 10 mM Tris. For DNA fragmentation assays, total DNA was extracted from *S. cerevisiae* (W303) treated cells as described previously with modifications (3). Briefly, cells were collected by centrifugation at 3500 rpm for 10 min, resuspended in lysis buffer and 0.5 mm zirconia beads (Biospec Products). DNA was extracted as described above. The pellet was resuspended in 10 mM Tris with 1 mg/ml RNase and incubated at 37°C for 0.5 h. Ethanol (95% v/v) and 3 M sodium acetate solution (pH 5.2) was added and the samples were stored at 20°C overnight. The DNA samples were analyzed by electrophoresis on a 1% agarose gel (at 60 V/30 mA). 1 kb DNA ladder (Invitrogen) was used as marker. The gel was visualized on a gel-doc system (Biorad) after staining with ethidium bromide.

Curing of nucleic acids

The killer yeasts were plated on YPD-agar plates at a density of 10^7 cells/plate and was subjected to ultraviolet light irradiation (254 nm) at a dose of 20,000 µJ/cm2 for 10 s (lethality about 80-98%) with a UV cross-linker (Stratalinker UV). The UV-irradiated plates were incubated at 30°C for 4 days. The presence or absence of plasmid was examined by electrophoresis of nucleic acids. As positive control for plasmid curing, we used a strain that contains a 4.5 kb double-stranded RNAs (dsRNAs) from (*S. cerevisiae* strain S288C) (28).

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

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).

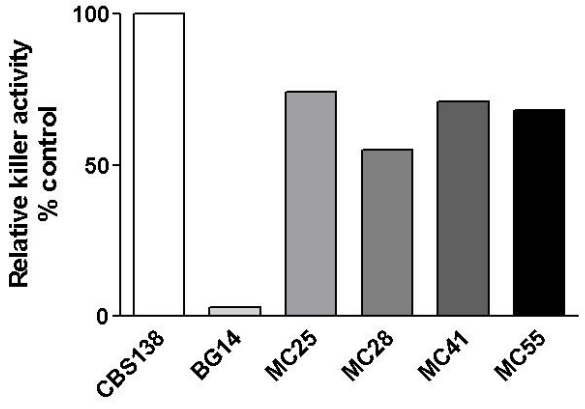

Figure 1. Killer activity of four *C. glabrata* clinical isolates. Killer yeast (CBS138), killer yeast negative (BG14). n=5.

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.

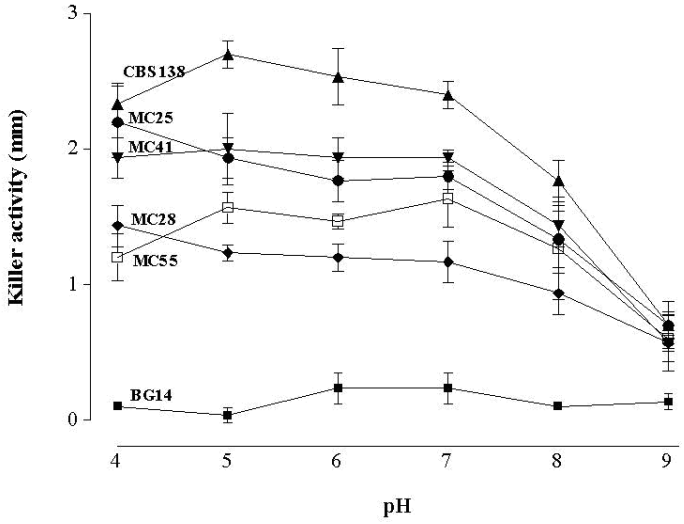

Figure 2. Effect of pH on stability of killer activity. Culture filtrates were adjusted to the appropriate pH (4-9) with 0.1 M citrate phosphate buffer and kept at 4°C. Determination of killer activity was assayed. n= 5.

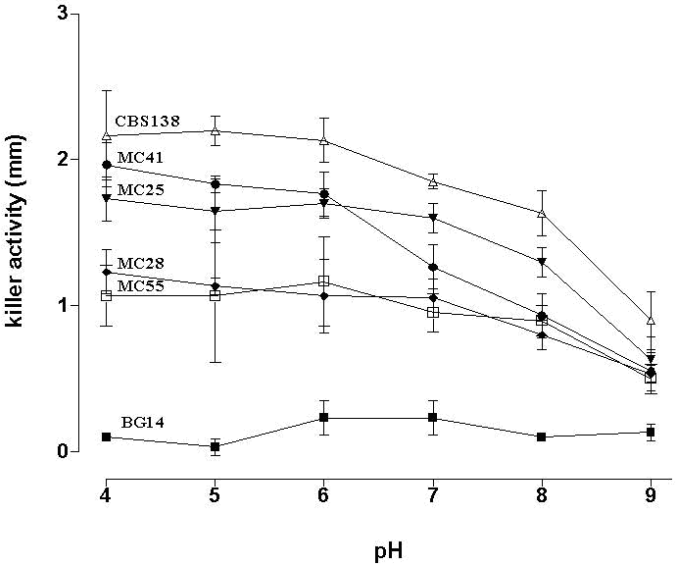

Figure 3. Effect of the pH in the *C. glabrata* growth media on the production of killer activity. YPD aliquots were adjusted to the appropriate pH values (4-9) with 0.1 M citrate phosphate buffer. Killer activity was assayed. n=4.

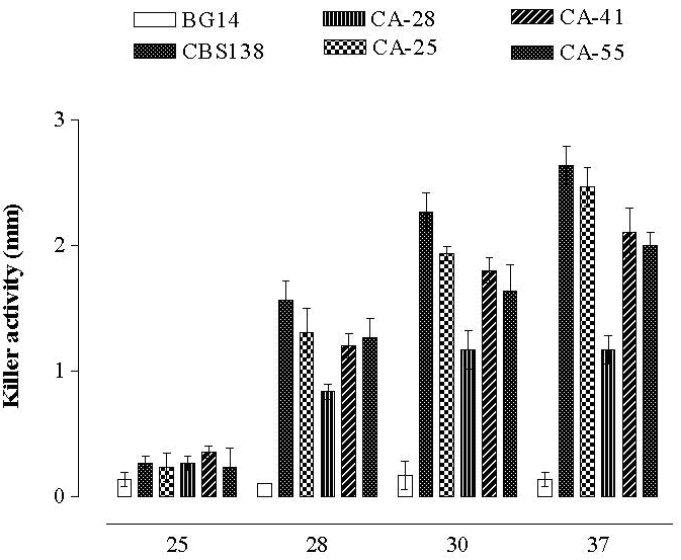

Figure 4. Temperature effect on killer activity. The killer activity was assayed using culture filtrates at pH 7.0 and incubated at 25, 28, 30 and 37 °C for 72 h. n=4.

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.

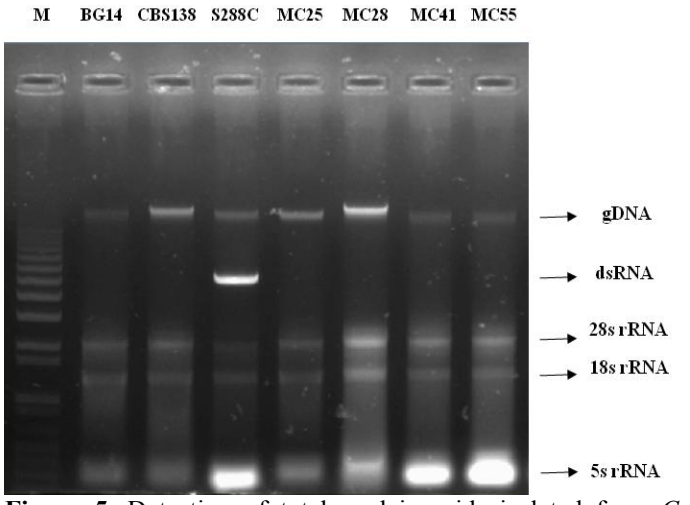

Figure 5. Detection of total nucleic acids isolated from *C. glabrata* clinical isolates by agarose gel electrophoresis. BG14 (killer negative), CBS138 (killer yeast), *S. cerevisiae* (contain a 4.5 kb dsRNA plasmid used as positive control). gDNA, genomic DNA. dsRNA, double stranded RNA. n=3.

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).

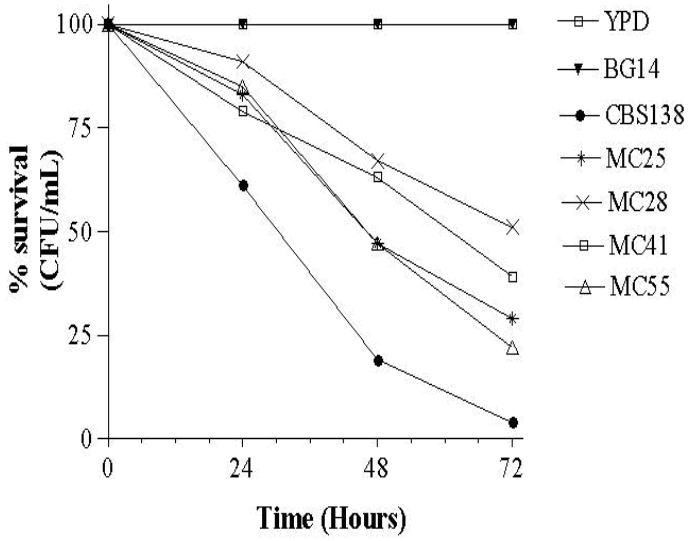

Figure 6. Sensitivity of *S. cerevisiae* to killer extract from *C. glabrata* clinical isolates. A, % survival (CFU %) of *S. cerevisiae* in liquid media incubated with killer extracts, log CFU (%) is the relative number of colony forming units; time 0= 5 x 10 6 cells. CFU was measured at intervals by diluting and plating on YPD. n= 5.

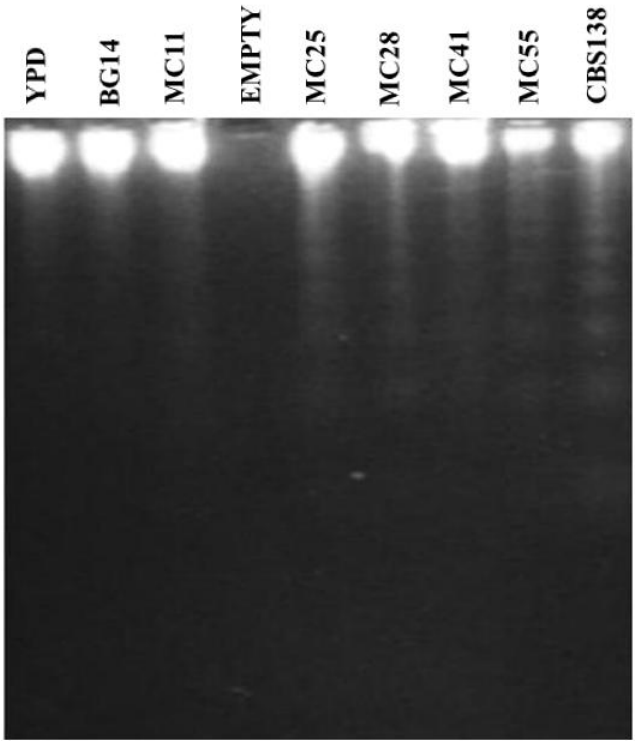

Figure 7. Survival of *S. cerevisiae* and DNA fragmentation induced by killer toxins from *C. glabrata* clinical isolates. Agarose electrophoresis with total DNA from *S. cerevisiae* treated with killer extracts for 72 h at 30°C. n=3.

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 H_2O_2, 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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