Vaginal isolates of *Candida glabrata* are Uniquely Susceptible to Killer Toxins Produced by *Saccharomyces* Yeasts

Lance R. Fredericks, Mark D. Lee, Cooper R. Roslund*, Mason A. Shipley*, Paul A. Rowley#

Department of Biological Sciences, University of Idaho, Moscow, ID, USA
*Authors contributed equally
#Correspondence: prowley@uidaho.edu

ABSTRACT

Killer toxins have a range of antifungal activities against pathogenic species of yeasts. *Candida glabrata* was found to be acutely susceptible to seven killer toxins produced by *Saccharomyces* species of yeasts. Specifically, the ionophoric K1 and K2 killer toxins were broadly inhibitory to a large collection of *C. glabrata* from patients with recurrent vulvovaginal candidiasis. The sensitivity of these clinical isolates to killer toxins does not correlate with their resistance to major classes of antifungal drugs.

RESEARCH FINDINGS

Vulvovaginal candidiasis (VVC) is estimated to afflict two in every three women worldwide at some point in their lives causing significant suffering and associated economic losses [1–3]. *Candida albicans* is most often isolated as the dominant species present in cases of VVC, followed by *Candida glabrata*, which is the second most prevalent cause of VVC [4]. Additionally, in certain diabetic patient populations *C. glabrata* is the dominant yeast species associated with VVC [5,6]. The frontline treatment for VVC is oral doses of fungistatic fluconazole. However, drug-resistant *Candida* yeasts can result in treatment failure and longer courses of suppressive treatment [7–9]. To relieve symptoms of VVC and to prevent *Candida*-associated complications during pregnancy, topical application of azoles is preferred over oral formulations of azole due to the potential for fetal toxicity during the first trimester [10]. However, the efficacy of azoles is reduced in the acidic environment of the vagina [11]. The limited availability of effective non-toxic therapies to treat VVC warrants the exploration of novel therapeutics that are active at the low pH of the vagina.

Killer toxins produced by *Saccharomyces* “killer” yeasts are optimally active at acidic pHs that overlap the average pH of the vaginal mucosa (pH ~4.5). In addition, there have been many publications that have described killer yeasts that can inhibit the growth of pathogenic yeasts [12–17]. Given the relatively recent discovery of several novel killer toxins produced by *Saccharomyces* yeasts, 15 species of pathogenic *Candida* yeasts were assayed for their susceptibility to the killer toxins K1, K1L, K2, K21/K66, K45, K62, and K74 [18–24]. A well assay was used to inoculate killer yeasts into pH 4.6 buffered agar plates seeded with lawns of pathogenic *Candida* yeasts (Figure S1). After three days of incubation at room temperature, growth inhibition was identified by the appearance of yeast-free zones and halos of oxidized methylene blue around killer yeasts. Of the 15 species of yeasts challenged, *C. glabrata* appeared to be the most susceptible to killer toxins produced by 6 of the 9 killer yeasts assayed,

followed by *C. nivarensis* and *C. bracarensis*, which are all human pathogenic species of the *Nakaseomyces* genus (Figure 1A and B).

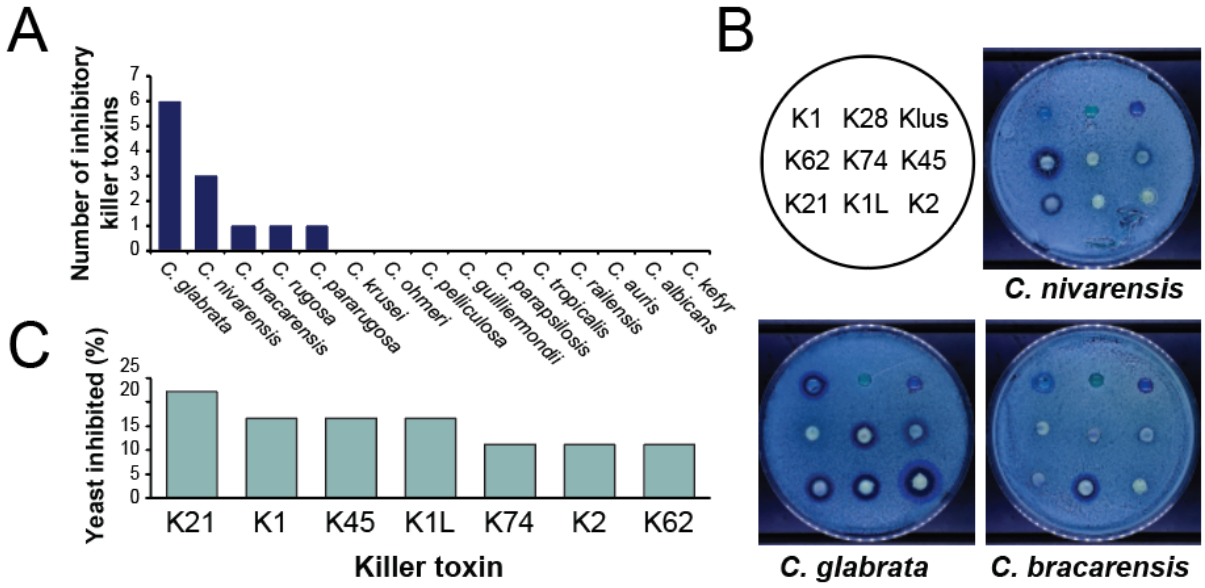

Figure 1. *Candida glabrata* is more susceptible to killer toxins than other species of *Candida* yeasts. (A) The number of killer yeasts that inhibit the growth of different species of pathogenic yeasts. (B) Representative agar plug assay plates with nine different types of killer yeasts seeded with *C. glabrata, C. nivarensis*, and *C. bracarensis*. (C) The percentage of *Candida* yeast species inhibited by each type of killer toxin.

As killer toxin susceptibility can vary widely within a species, the unique sensitivity of *C. glabrata* to nine different killer toxins was further investigated in 53 primary clinical isolates from the human vagina. Killer yeasts were pinned onto agar plates (YPD, pH 4.6) seeded with lawns of *C. glabrata* and qualitatively assayed for growth inhibition and methylene blue straining. Of the 477 interactions measured between killer yeasts and *C. glabrata*, it was observed that killer yeasts producing the ionophoric killer toxins K1, K2, and K45 were inhibitory to 100%, 96%, and 75% of *C. glabrata* isolates, respectively (Figure 2A). The remaining killer toxins affected the growth and viability of less than 33% of isolates. Curiously, a recently identified homolog of K1 named K1L was only able to inhibit the growth of 32% of clinical isolates suggesting that *C. glabrata* is not broadly susceptible to all ionophoric killer toxins. The susceptibility of *C. glabrata* to K1 and K2 greatly contrasts the widespread killer toxin resistance observed by different strains of *Saccharomyces* yeasts (Figure 2A). To test the susceptibility of clinical isolates of *C. glabrata* with acute killer toxin exposure, K1 and K2 toxins were enriched from spent culture media by ammonium sulfate precipitation [24]. Application of toxins to lawns seeded with 1×10^5 of *C. glabrata* yeast cells demonstrated their concentration-dependent susceptibility by measuring the zone of growth inhibition and methylene blue staining (Figure 2B). The mean antifungal activity of K1 with 95% confidence was typified by both zones of growth inhibition (with 95% confidence) (92.34 mm^2 ±11.24) and halos of methylene blue staining (41.44 mm^2 ± 4.98), and K2 resulted in zones of growth inhibition (58.48 mm^2 ± 11.91) and large zones of methylene blue staining (185.68 mm^2 ± 28.66) (Figure 2B). Importantly, we observe only a weak correlation (Spearman's correlation coefficient $R^2 = 0.31$) between inhibition of *C. glabrata* by K1

and K2, indicating different mechanisms of intoxication (Figure S2). Using the Kirby-Bauer disk diffusion assay to measure mean growth inhibition (in mm^2 with 95% confidence), it was determined that many of the clinical isolates of *C. glabrata* had variable resistance to the antifungal drugs fluconazole (1.21 mm^2 ± 1.06), ketoconazole (18.78 mm^2 ± 1.67) clotrimazole (5.82 mm^2 ± 1.30), amphotericin B (11.24 mm^2 ± 0.21), and caspofungin (25.25 mm^2 ± 0.29) (Figure S3). The observed variation in the inhibition of *C. glabrata* by K1 and K2 in Figure 2B did not correlate with the varying susceptibility of clinical isolates to these antifungal drugs (Figure 2C).

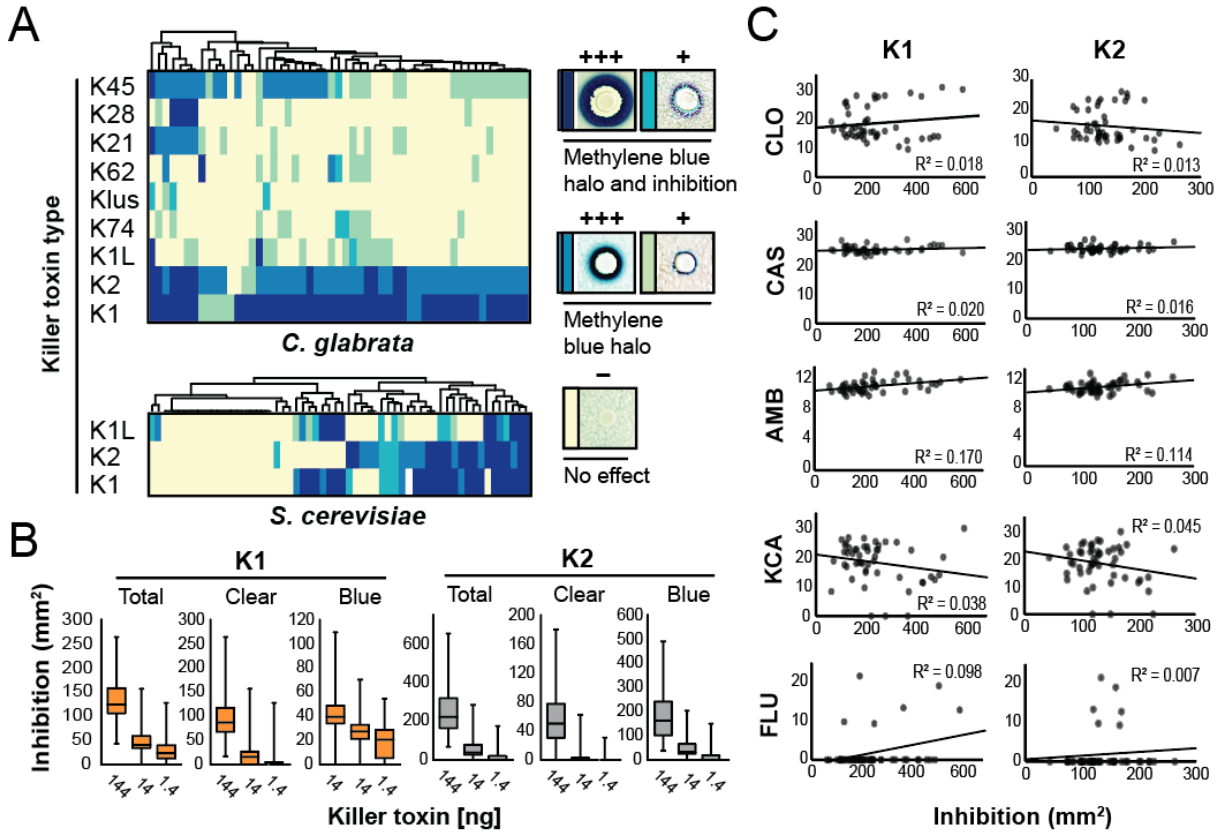

Figure 2. Clinical isolates of *C. glabrata* from the vaginal mucosa are most susceptible to the ionophoric killer toxins K1 and K2. (A) Cluster analysis of the susceptibility of 53 isolates of *C. glabrata* and *S. cerevisiae* to nine types of killer toxin produced by *Saccharomyces* yeasts as assayed on agar plates. (B) Box and whisker plot showing the median killer toxin activity based on the size of the total area of inhibition, zones of complete growth inhibition (clear) or methylene blue staining (blue) around killer yeasts for three concentrations of K1 and K2 killer toxins. (C) Spearman's correlation analysis of the relationship between the susceptibility of *C. glabrata* to killer toxins and the clinical antifungal drugs clotrimazole (CLO, 50 μg), caspofungin (CAS, 5 μg), amphotericin B (AMB, 20 μg), ketoconazole (KCA, 15 μg), and fluconazole (FLU, 25 μg).

CONCLUSION

We have found that *C. glabrata* is susceptible to a diversity of killer toxins produced by *Saccharomyces* yeasts and that K1 and K2 are broadly antifungal to primary clinical isolates from the vaginal mucosa. This work updates our understanding of the interaction of killer toxins with pathogenic species of *Candida* yeasts and demonstrates the value of screening multiple isolates of a pathogenic species to identify broad-spectrum antifungal activities. The antifungal

mechanism of K1 and K2 are both thought to involve the attack of the fungal membrane and ion leakage [25]. Thus, we expect that the plasma membrane is the site of K1 and K2 action against *C. glabrata* resulting in cell lysis and apoptosis [26]. Further work will be required to confirm the mechanism of action of ionophoric toxins against *C. glabrata* and to explore the molecular determinants of the observed unique susceptibility.

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SUPPLEMENTAL FIGURES

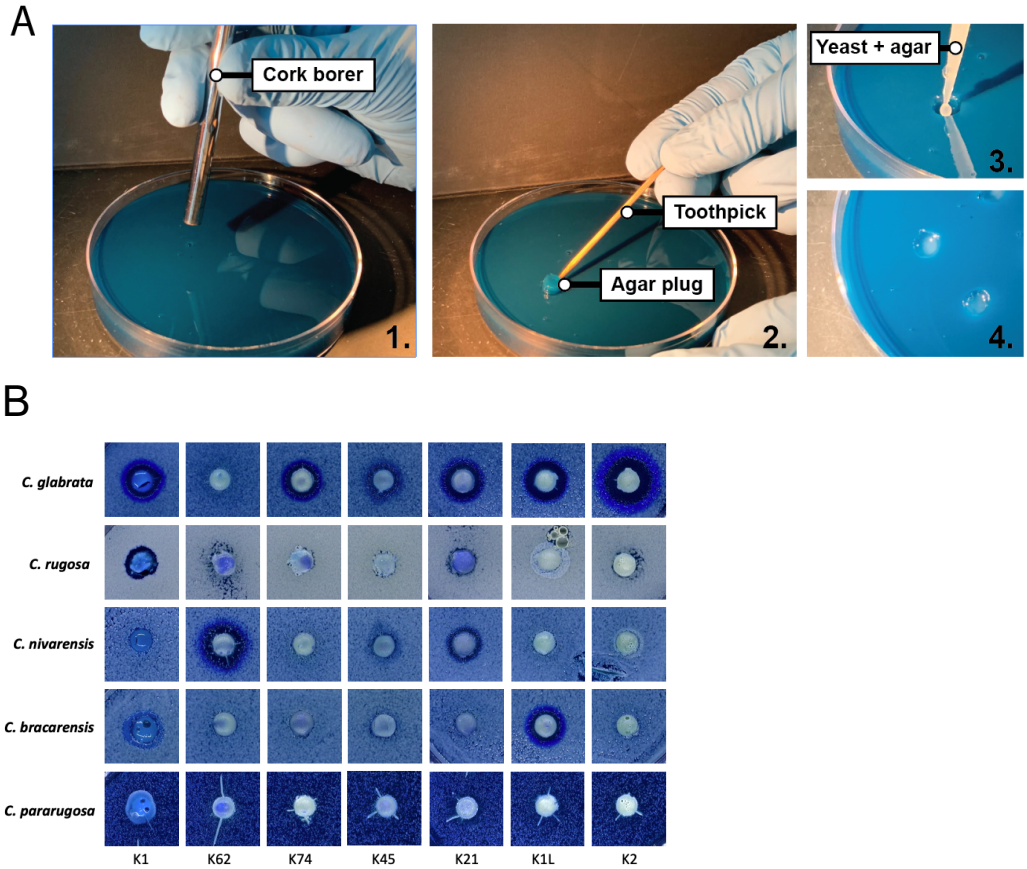

Figure S1. Assay of 15 species of pathogenic yeasts against seven different types of killer toxin using a well plate assay. (A) Well assay method overview 1) Wells were cut in an agar plate (YPD pH 4.6 with methylene blue) that was previously seeded with ~1x10e5 pathogenic yeast using a sterilized cork borer (6 mm diameter). 2) Wells were excavated using a sterile toothpick to remove and discard the agar plug. 3) Approximately 5 mg of yeast from an overnight culture is mixed to a final volume of 200 uL with molten agar and pipetted into each well. 4) Agar was left to set and the plate was incubated for three days at room temperature. (B) Representative images of the inhibition of pathogenic *Candida* yeasts by *Saccharomyces* killer yeasts using the well assay.

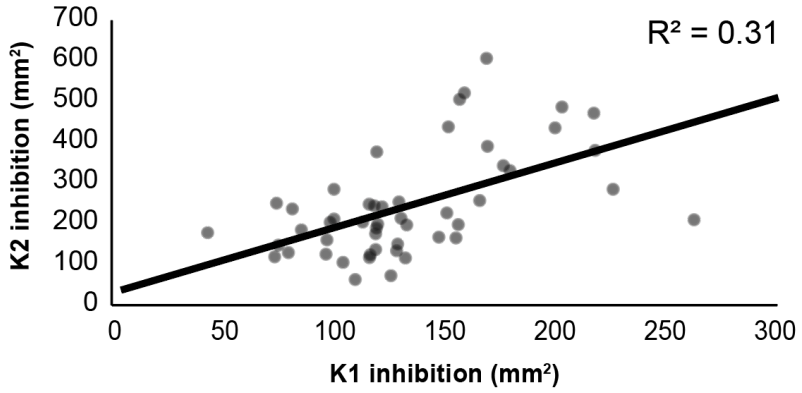

Figure S2. A weak correlation between the extent of *C. glabrata* inhibition by K1 and K2.

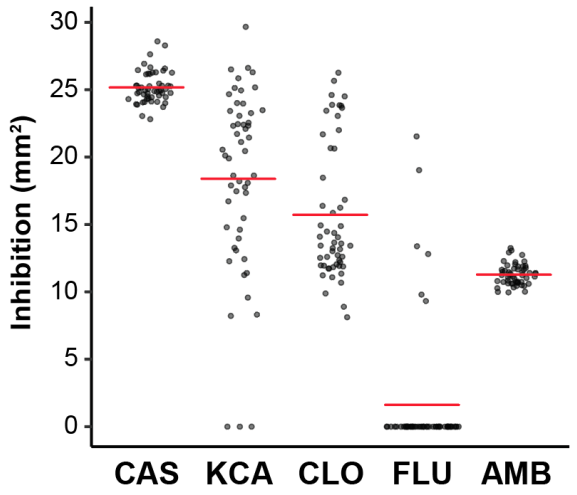

Figure S3. The susceptibility of clinical isolates of *C. glabrata* to clinically relevant antifungal drugs. A Areas of growth inhibition were measured by the disk diffusion assay and the total area of growth inhibition was measured by calipers. Horizontal red bars represent the average area of inhibition. Antifungals assayed: caspofungin (CAS, 5 μg), ketoconazole (KCA, 15 μg), clotrimazole (CLO, 50 μg) fluconazole (FLU, 25 μg) and amphotericin B (AMB, 20 μg).

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