Vaginal Isolates of *Candida glabrata* are Uniquely Susceptible to Ionophoric Killer Toxins Produced by *Saccharomyces cerevisiae.*

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ABSTRACT

Compared to other species of *Candida* yeasts, the growth of *Candida glabrata* was inhibited by many different strains of *Saccharomyces* killer yeasts. The ionophoric K1 and K2 killer toxins were broadly inhibitory to all clinical isolates of *C. glabrata* from patients with recurrent vulvovaginal candidiasis, despite high levels of resistance to clinically relevant antifungal therapeutics.

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* (4). In certain diabetic patient populations, *C. glabrata* can be the dominant yeast species associated with VVC (5, 6). The main treatment for VVC is the orally administered fungistatic azole, fluconazole. During pregnancy, to relieve symptoms of VVC and to prevent *Candida*-associated complications, topical application of azoles is preferred over oral

25 administration due to the potential for fetal toxicity in the first trimester (7). However, drug-
26 resistance in isolates of *C. glabrata* and other species of *Candida* yeasts is increasing and can
27 result in long courses of suppressive treatment and treatment failure (8–10). The limited
28 availability of effective non-toxic therapies to treat VVC warrants the exploration of novel
29 therapeutics that are active at the normal low pH of the vagina.
30
31 Killer toxins produced by *Saccharomyces* "killer" yeasts are optimally active in acidic conditions
32 (pH $\leq$4.6) that overlap the pH of the vaginal mucosa (~pH 4.2) (11). In addition, there have been
33 many studies describing killer yeasts that can inhibit the growth of pathogenic fungi (12–17).
34 Given the discovery of novel killer toxins produced by *Saccharomyces* killer yeasts, 16 species
35 of the *Candida* genus were screened for their susceptibility to nine killer yeast strains known to
36 express K1, K1L, K2, K21/K66, K28, K45, K62, K74, or Klus killer toxins encoded by double-
37 stranded satellite RNAs (18–24). To test the ability of different killer yeasts to inhibit the growth
38 of *Candida* yeasts, a well assay was used to inoculate killer yeasts into killer assay agar plates
39 (YPD agar plates with 0.003% w/v methylene blue buffered to pH 4.6 with sodium citrate (25))
40 seeded with ~1×10^5 *Candida* yeast cells (Fig. S1). After three days of incubation at room
41 temperature, growth inhibition was identified by the appearance of yeast-free zones and/or
42 halos of oxidized methylene blue around killer yeasts (Fig. 1 and S1). Of the 16 species of
43 *Candida* yeasts challenged, *C. glabrata* appeared to be the most susceptible to killer yeasts and
44 was only resistant to the killer yeast that expressed K62 (Fig. S1 and Table S1). One additional
45 species of the *Nakaseomyces* genus (*C. nivariensis*) was also more susceptible to
46 *Saccharomyces* killer yeasts than other *Candida* yeasts (Fig. 1A and B). The K21 killer yeast
47 appeared to have the broadest spectrum of antifungal activity (Fig. 1C).
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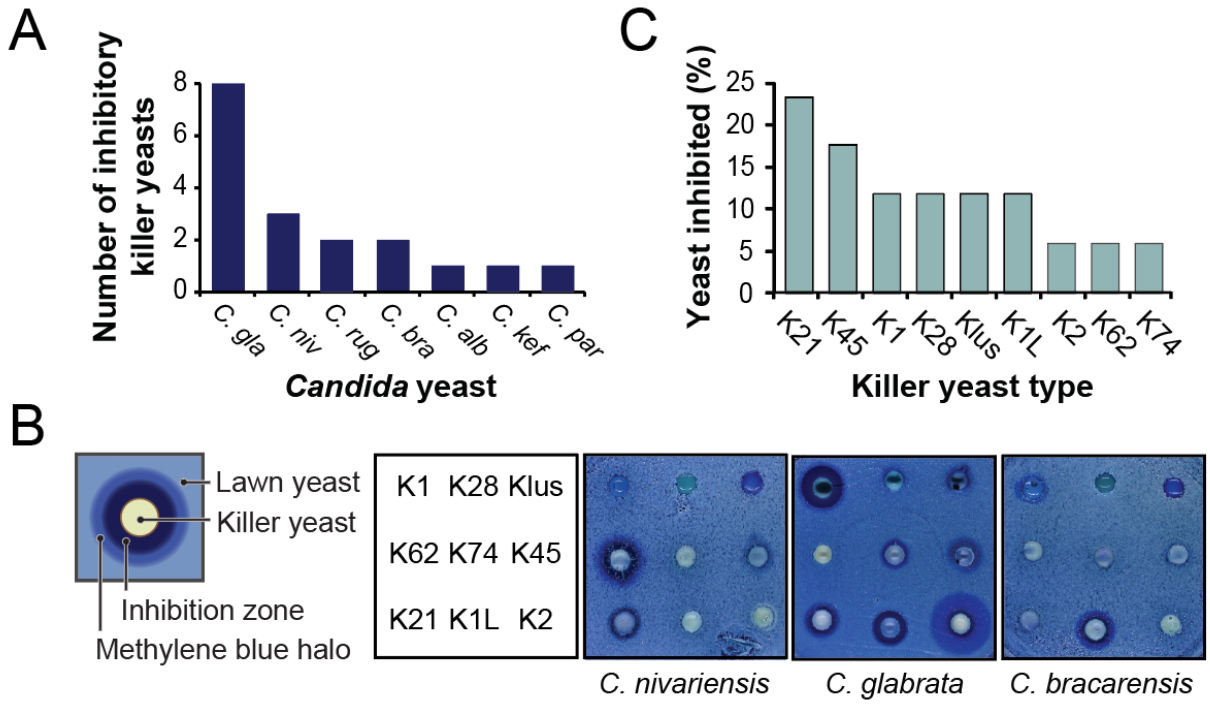

Fig. 1. *Candida glabrata* is more susceptible to inhibition by killer yeasts than other species of *Candida* yeasts. (A) The number of killer yeasts that inhibit the growth of different species of *Candida* yeasts; *C. glabrata* (*C. gla*), *C. nivariensis* (*C. niv*), *C. kefyr* (*C. kef*), *C. bracarensis* (*C. bra*), *C. rugosa* (*C. rug*), *C. pararugosa* (*C. par*), and *C. albicans* (*C. alb*) (n = 2). (B) A schematic illustration of the effect of a killer yeast on the growth of a competing lawn of yeast. Representative well assay plates with nine different killer yeasts on agar seeded with representative species of *Candida* yeasts. (C) The percentage of *Candida* yeast species found to be inhibited by each type of killer toxin (n = 2).

As killer toxin susceptibility can vary widely within a species, 53 unselected clinical isolates of *C. glabrata* from the human vagina were challenged by killer yeasts. These clinical isolates were collected at the Wayne State University Vulvovaginitis clinic in Detroit, MI, between 2015 and 2019. Killer yeasts were inoculated onto the surface of killer assay agar plates seeded with lawns of *C. glabrata* and qualitatively assayed for evidence of growth inhibition. Of the 477 interactions measured between killer yeasts and *C. glabrata*, K1, K2, and K45 killer yeasts inhibited the growth of 100%, 96%, and 75% of *C. glabrata* isolates, respectively (Fig. 2A, top).

67 The remaining killer yeasts each inhibited less than 33% of *C. glabrata* isolates. The
68 susceptibility of *C. glabrata* to K1 and K2 killer yeasts greatly contrasts the widespread
69 resistance of *Saccharomyces* yeasts (Fig. 2A, bottom). To test the susceptibility of the clinical
70 isolates of *C. glabrata* with acute killer toxin exposure, K1 and K2 toxins were partially purified
71 from 1 mL of spent culture medium, as described previously (23). Exposure of lawns seeded
72 with ~1×10^5 of *C. glabrata* cells to K1 or K2 demonstrated a concentration-dependent growth
73 inhibition of *C. glabrata* (Fig. 2B). All isolates of *C. glabrata* were inhibited by K1 and K2 at the
74 highest concentration tested, with K2 exposure resulting in large halos of methylene blue
75 (185.68 mm^2 ± 28.66; 95% CI) whereas K1 produced larger zones of growth inhibition (92.34
76 mm^2 ±11.24; 95% CI) (Fig. 2B). The inhibition of *C. glabrata* is similar to the fungicidal effects of
77 K1 and K2 toxins against susceptible strains of *S. cerevisiae* (Fig. S2). Neither K1 nor K2 were
78 acutely cytotoxic to cultured human cells at physiological pH as measured by an alamar blue
79 viability assay (Fig. S3). Both K1 and K2 retained measurable activity against yeast cells after a
80 1 hour incubation with human epithelial cells (HeLa cell line) in Dulbecco's modified eagle
81 medium with 10% serum at pH 7 (Fig. S3).
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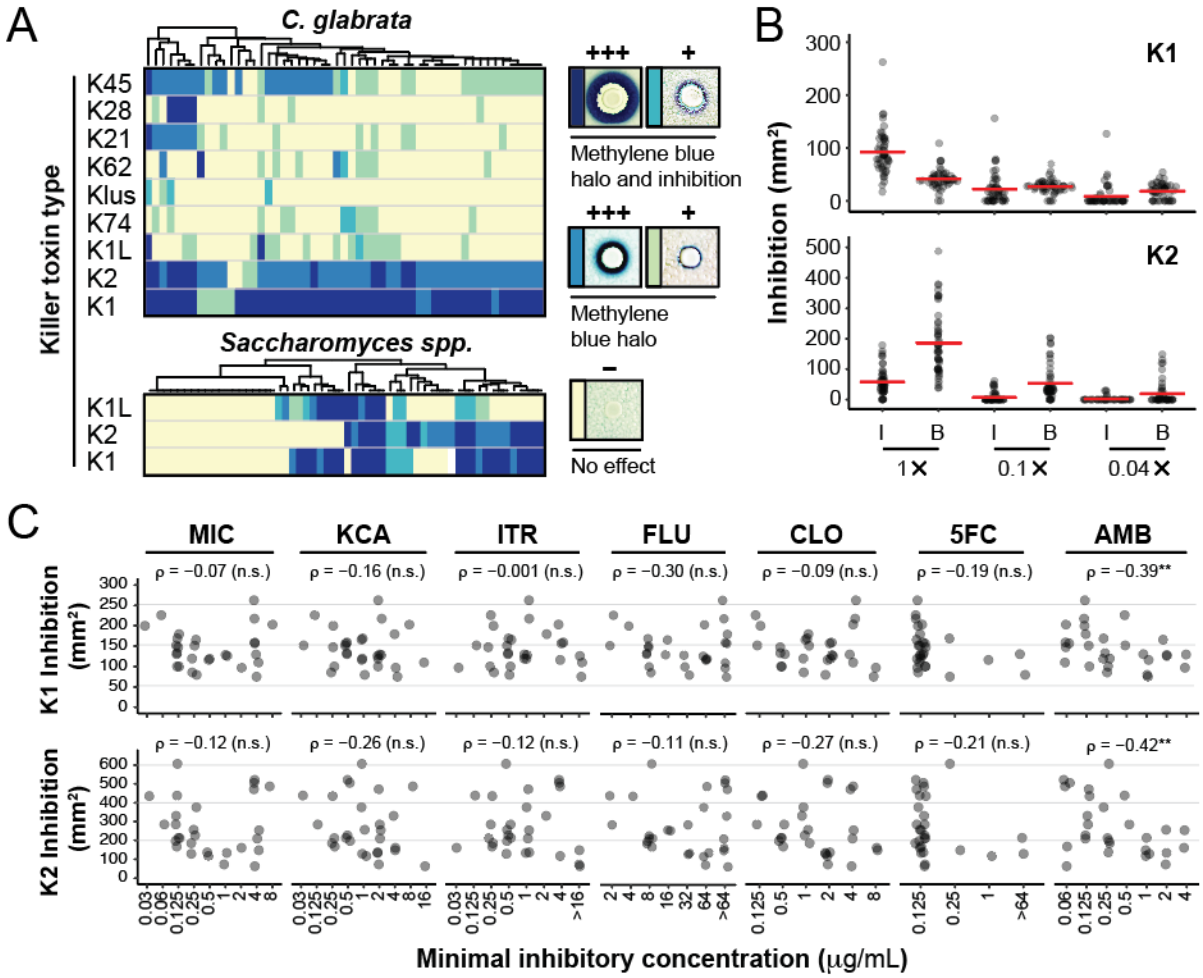

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85 **Fig. 2. Drug resistant clinical isolates of *C. glabrata* from the vagina are most susceptible to the ionophoric**
86 **killer toxins K1 and K2.** (A) Cluster analysis of the susceptibility of 53 isolates of *C. glabrata* (top) and 53 strains of
87 *Saccharomyces* species (bottom) to different types of killer yeasts as assayed on agar plates. (B) The susceptibility of
88 50 isolates of *C. glabrata* to partially purified K1 and K2 killer toxins showing the mean killer toxin activity based on
89 the area of complete growth inhibition (I) or methylene blue staining (B) on agar. The 1× concentration of K1 used
90 was 13 μg/mL as measured by Western blotting using a custom antibody raised to a K1 derived peptide. (C) The
91 minimum inhibitory concentration of seven antifungal drugs against 27 Wayne State clinical isolates of *C. glabrata*
92 compared to the total area (methylene blue staining and zone of growth inhibition) of cytotoxicity caused by K1 or K2
93 killer toxins. Correlation and significance values were calculated by Spearman's rank correlation analysis (n.s. = not
94 significant, **p<0.05). Antifungal drugs assayed: fluconazole (FLU), clotrimazole (CLO), ketoconazole (KCA),
95 miconazole (MIC), itraconazole (ITR), amphotericin B (AMB), and flucytosine (5FC).

96

97 Clinical isolates of *C. glabrata* were found to vary in their resistance to antifungals used to treat
98 both VVC and invasive candidemia using the NCCLS M27-A method to calculate MIC values
99 (Fig. 2C) and the disk diffusion assay (Fig. S2). Even when *C. glabrata* isolates were highly
100 resistant to clinical antifungals they remained susceptible to acute K1 and K2 exposure. There
101 was no significant correlation between drug resistance and killer toxin susceptibility (Fig. 2C and
102 S2), except a weak correlation between K1 and K2 resistance and amphotericin B resistance
103 ($p<0.05$) (Fig. 2C and S2).
104
105 Killer toxins are notoriously strain- and species-specific to the point that they have been used to
106 identify different strains of pathogenic yeasts (26). However, the data presented in this study
107 show that most types of known *Saccharomyces* killer yeasts can inhibit the growth of *C.*
108 *glabrata.* Specifically, the qualitative screening of killer yeasts identified that the ionophoric
109 toxins K1 and K2 were broadly inhibitory to vaginal isolates of *C. glabrata* and that purified
110 toxins inhibited growth in a concentration-dependent manner. The *C. glabrata* cell wall is
111 structurally similar to *S. cerevisiae* and this would suggest that K1 and K2 bind the *C. glabrata*
112 cell wall β-1,6 glucan as the primary receptor (27). *C. glabrata* also expresses a homolog of *S.*
113 *cerevisiae* Kre1p, which is the GPI-anchored secondary membrane receptor used by both K1
114 and K2 that enables membrane attack (28). The mechanism of *C. glabrata* intoxication is likely
115 to involve the disruption of ion homeostasis by pore formation in the plasma membrane, as has
116 been shown for *S. cerevisiae* (29). However, cell wall binding and the presence of Kre1p are not
117 sufficient for intoxication, as K1 is able to bind the cell wall and utilize Kre1p of *C. albicans*,
118 which is intrinsically K1 resistant (30, 31). Furthermore, it is unclear why other species of the
119 *Nakaseomyces* genus (*C. nivariensis* and *C. bracarensis*) that are closely related to *C. glabrata*
120 are more resistant to inhibition by the same killer yeasts.
121

122 The alteration of ergosterol biosynthesis can cause resistance to azoles and amphotericin B in
123 *Candida* yeasts (32–35), and we find that the latter significantly correlates with increased K1
124 and K2 resistance (Fig. 2C). As amphotericin resistance can be caused by a reduction in the
125 concentration of membrane ergosterol, increased K1 and K2 resistance in *C. glabrata* could be
126 due to alterations in the composition, fluidity, and permeability of the yeast plasma membrane.
127 Similar protection from K1 intoxication is observed in strains of *S. cerevisiae* with defects in
128 ergosterol biosynthesis (36). Natural K1 resistant strains of *S. cerevisiae* also have a lower
129 expression of ergosterol biosynthesis genes and a reduced concentration of ergosterol esters
130 (37). The depletion of membrane sterols can result in resistance to many cytotoxic proteins (38–
131 42). Moreover, sterol-rich membrane microdomains (lipid rafts) provide a nucleation point for
132 protein toxins that bind raft-localized receptors (43–45). The depletion or redistribution of
133 cholesterol can disrupt raft integrity and inhibit the binding of toxins to their cognate receptor
134 (43, 46). Thus, susceptibility of *C. glabrata* to K1 and K2 could be influenced by membrane
135 ergosterol and the localization and function of the GPI-anchored secondary membrane receptor,
136 Kre1p.
137
138 The screening of killer yeasts has served to identify the unique activity of K1 and K2 against *C.*
139 *glabrata* suggesting that they could be useful as novel antifungal agents. Compared to azoles,
140 these killer toxins are fungicidal, optimally active at low pH, and are non-toxic to human cells at
141 physiological pH(28, 47–49). Therefore, we speculate that with further mechanistic studies,
142 formulation and stabilization, K1 and K2 could be developed for topical application to combat *C.*
143 *glabrata* associated with VVC.
144
145
146 **Acknowledgments**

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