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Research Article

# The prevalence of killer yeasts and double-stranded RNAs in the budding yeast *Saccharomyces cerevisiae*

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## Abstract

Killer toxins are antifungal proteins produced by many species of "killer" yeasts, including the brewer's and baker's yeast *Saccharomyces cerevisiae*. Screening 1270 strains of *S. cerevisiae* for killer toxin production found that 50% are killer yeasts, with a higher prevalence of yeasts isolated from human clinical samples and winemaking processes. Since many killer toxins are encoded by satellite double-stranded RNAs (dsRNAs) associated with mycoviruses, *S. cerevisiae* strains were also assayed for the presence of dsRNAs. This screen identified that 51% of strains contained dsRNAs from the mycovirus families *Totiviridae* and *Partitiviridae*, as well as satellite dsRNAs. Killer toxin production was correlated with the presence of satellite dsRNAs but not mycoviruses. However, in most killer yeasts, whole genome analysis identified the killer toxin gene *KHS1* as significantly associated with killer toxin production. Most killer yeasts had unique spectrums of antifungal activities compared to canonical killer toxins, and sequence analysis identified mutations that altered their antifungal activities. The prevalence of mycoviruses and killer toxins in *S. cerevisiae* is important because of their known impact on yeast fitness, with implications for academic research and industrial application of this yeast species.

**Keywords:** mycovirus; dsRNA satellite; killer toxin; killer phenotype; killer yeast

## Introduction

Since the discovery of antifungal toxins produced by "killer" yeasts (Bevan and Makower 1963), many studies have sought to identify "killer" toxins that can inhibit the growth of different strains and species of fungi (such as (Philliskirk and Young 1974, Stumm et al. 1977, Middelbeek et al. 1980, Starmer et al. 1987, Yap et al. 2000, Nakayashiki et al. 2005, Baeza et al. 2008, Pieczynska et al. 2013)). The broad interest in killer yeasts has been driven by their ability to inhibit the growth of pathogenic and spoilage fungi that are important for the health of humans and plants. Moreover, there have been successful demonstrations of the application of killer toxins to prevent disease and spoilage in agriculture (such as (Kitamoto et al. 1993, Santos 2004, Liu and Tsao 2009, Schnürer and Jonsson 2010, Haïssam 2011, Perez et al. 2016, Díaz et al. 2020)). This includes creating transgenic plants that express killer toxins to prevent disease (Clausen et al. 2000). *S. cerevisiae* killer toxins can also be applied to prevent the growth of unwanted yeasts during brewing and winemaking (Gutiérrez et al. 2001, Zhong et al. 2022). Moreover, the opportunistic human pathogen *Candida glabrata* appears to be particularly sensitive to *S. cerevisiae* killer toxin K1 (Bussey and Skipper 1976, Walker et al. 1995, Fredericks et al. 2021b). In general, killer toxins have been proposed to be important for interference competition based on the observation that killer yeasts can outcompete and invade toxin-susceptible yeast populations (Pintar and Starmer 2003, Greig and Travisano 2008, McBride et al. 2008, Wloch-Salamon et al. 2008). Moreover, the production of killer toxins can influence yeast community composition and insect interactions (Ganter and Starmer 1992, Buser et al. 2021). However, the spectrum of antifungal activity of *S. cerevisiae* killer yeasts is mainly limited to closely related strains and species of yeasts (Stumm et al. 1977, Santos et al. 2004, Ullivarri et al. 2014, Fredericks et al. 2021a). Although many killer yeasts have been discovered, no definitive studies have comprehensively investigated their prevalence and diversity across any fungal species.

There are 11 known killer toxin types produced by *S. cerevisiae* (K1, K2, K28, Klus, KHR, and KHS) and *Saccharomyces paradoxus* (K1L, K21/K66, K45, K62, and K74). These toxins are unrelated by sequence homology, except for K1 and K1L (Fredericks et al. 2021a). Of these killer toxins, the mechanisms of action have been studied best in K1 and K28. K1 has been characterized as an ionophoric toxin that causes unregulated potassium efflux from susceptible cells, likely by pore formation in the fungal plasma membrane that requires the GPI-anchor protein Kre1 as a membrane receptor (Martinac et al. 1990, Breinig et al. 2002). Similar ionophoric modes of action have been proposed for other killer toxins such as K1L, K2, Zygocin, and *Pichia kluyveri* killer toxin (Kagan 1983, Weiler and Schmitt 2003, Orentaite et al. 2016, Fredericks et al. 2021a). K28 interacts with the membrane H/KDEL receptor Erd1 and is endocytosed before retrograde transport to the cytoplasm (Becker and Schmitt 2017). The small size of K28 then enables diffusion to the nucleus, leading to G1/S cell cycle arrest and cell death by an unknown mechanism (Schmitt et al. 1996). Like many killer toxins produced by other species of yeasts, these toxins are most active in acidic conditions (≈pH 4.6) at temperatures below 30°C (Woods and Bevan 1968, McBride et al. 2008). The

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conditions required for the optimal activity of killer toxins suggest they are most relevant for interference competition in environments such as fruit and cactus rots and human fermentations (i.e. winemaking).

Detection of killer toxin production requires using toxin-susceptible strains to observe growth inhibition. However, the number and choice of strains used for identifying killer yeasts have varied widely between studies. For example, several large-scale screens have characterized killer toxin production as a rare phenotype (Nakayashiki et al. 2005, Pieczynska et al. 2013, Chang et al. 2015). These studies used only limited numbers of strains to detect killer toxin production, which likely led to an underestimation of the numbers of *S. cerevisiae* killer yeasts. In contrast, up to 90% of *S. cerevisiae* winemaking strains can produce killer toxins (Kitano et al. 1984, Shimizu et al. 1985, Heard and Fleet 1987, Hidalgo and Flores 1994, Maqueda et al. 2012). A more recent study screened 100 diverse strains of *S. cerevisiae* using molecular and microbiological techniques and determined that 70% of strains produced killer toxins (Vijayraghavan et al. 2023). These studies have shown that the prevalence of killer yeasts varies depending on the methods used to detect killer toxin production and the ecological niche.

In *Saccharomyces* yeasts, KHR and KHS killer toxins are chromosomally encoded and present in many strains of *S. cerevisiae* and *S. paradoxus* (Goto et al. 1990, 1991, Vijayraghavan et al. 2023); they have also been identified in other species of fungi (Frank and Wolfe 2009, Cheeseman et al. 2014). Overall, the majority of killer toxins described in fungi appear to be chromosomally encoded. However, most *Saccharomyces* killer toxins depend on the presence of fungal viruses (mycoviruses) from the family *Totiviridae* (Wickner et al. 2013). Totiviruses act as helper viruses to replicate and package small (<3 kbp) non-autonomous satellite dsRNAs that only encode killer toxin genes. These dsRNA elements do not have an extracellular stage to their lifecycle and are transmitted vertically by cell division and cell-to-cell fusion during mating. Killer toxin production by *S. cerevisiae* is often used to infer the presence of low molecular weight satellite dsRNAs. However, non-killer yeasts can harbor other non-autonomous dsRNAs, including satellite viruses (X-dsRNAs) and satellite dsRNAs that encode non-functional killer toxin genes (S-dsRNAs), as well as bipartite partitiviruses (Esteban and Wickner 1988, Buskirk et al. 2020, Taggart et al. 2023). The strong association between killer yeasts and satellite dsRNAs in yeasts has aided the sequencing, cloning, and expression of many killer toxins (such as (Skipper et al. 1984, Russell et al. 1997, Rodríguez-Cousiño et al. 2011, Fredericks et al. 2021a)).

Without killer toxin production, mycoviruses have primarily been identified by extracting bulk cellular RNAs for short-read sequencing or by purifying and visualizing dsRNAs using agarose gels. These approaches have identified mycoviruses in nearly all of the major lineages of fungi (Myers et al. 2020). Mycoviruses of the families *Totiviridae*, *Narnaviridae*, and, more recently, the *Partitiviridae* have been identified in *S. cerevisiae* (Wickner et al. 2013, Taggart et al. 2023). Mycovirus screens have found that ≈20% of strains harbor narnaviruses (López et al. 2002, Nakayashiki et al. 2005), and totiviruses are present in 30–100% of strains, depending on the study (Nakayashiki et al. 2005, Vijayraghavan et al. 2022). As seen with killer yeasts, the prevalence of mycoviruses can be biased to specific ecological niches; for example, 95% of partitiviruses are associated with *S. cerevisiae* strains isolated from coffee and cacao beans (Taggart et al. 2023). Identifying the presence of mycoviruses in fungi is important because they can positively or negatively affect host fitness, which has been reported widely in many species of fungi (such as (Nuss 2005, Márquez et al. 2007, Vainio et al. 2017, Lau et al. 2018, Chau et al. 2023)).

The availability of well-characterized collections of *S. cerevisiae* strains isolated from across the globe offers a unique opportunity, for the first time, to determine the prevalence of killer yeasts, dsRNA mycoviruses, and satellite dsRNAs across a single fungal species. This study describes the screening of *S. cerevisiae* strains representing the global diversity of *S. cerevisiae* and has found that killer toxins are produced in 50.2% of strains assayed. Killer yeast identification depended on the strains used to screen for killer toxin production and was closely correlated with the presence of totiviruses and satellite dsRNAs. The unexpectedly high prevalence of killer toxin production in strains lacking dsRNAs was consistent with the presence of the genome-encoded killer toxin gene *KHS1*.

## Materials and methods

### Growth and maintenance of yeasts

All strains of yeasts used in this study were obtained from existing strain collections (National Collection of Yeast Cultures (NCYC), Fungal Genome Stock Center (FGSC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ), National BioResource Project NBRP), Complutense Yeast Collection, Complutense University of Madrid (CYC), and Agricultural Research Service culture collection (Northern Regional Research Laboratory) (ARS culture collection (NRRL)), and other laboratory-specific collections and were maintained on yeast extract peptone dextrose (YPD) agar medium (Strope et al. 2015, Ludlow et al. 2016, Peter et al. 2018).

### Killer yeast assay

Using the replicate function of the Singer Instruments ROTOR HDA, 96 well YPD-glycerol stock plates of *S. cerevisiae* were printed onto Plusplates containing YPD agar. Plates were incubated at room temperature for 48 h. The printed strains were used to challenge 12 killer toxin susceptible yeast strains seeded onto solid YPD "killer assay" agar (0.003% w/v methylene blue, buffered with sodium citrate to pH 4.6). Sensitive lawns were chosen based on their unique susceptibilities to canonical killer toxins. To prepare sensitive yeast lawns, each strain of killer toxin susceptible yeast was inoculated in 2 mL YPD broth for 16 h at 25 °C with shaking at 250 rpm. The overnight cultures were centrifuged at 3 000 × *g* for 5 min, and the cell pellet was suspended in sterile water. Each culture was then normalized to an $OD_{600}$ of 5.0 and diluted 1:500 (strains NCYC 777, Ms300C, NBRC 1815, CYC 1058, BY4741, Y-27106, Y-5509, DSM70459, and Y-1088), 1:250 (strains NBRC 1802, Y-2046), or 1:100 (strain NCYC 1006) for optimal lawn density. 1.5 mL of each dilution was applied to Plusplates filled with killer assay YPD agar using a sterile plastic spreader and allowed to dry. Arrayed *S. cerevisiae* strains on YPD agar were pinned onto the yeast-seeded lawns using the ROTOR HDA at a density of 96 strains per plate. Plates were incubated at ambient temperature for 4–7 days until growth inhibition of the susceptible yeast lawn could be observed as a methylene blue halo and a zone of complete growth inhibition (Fig. 1A).

### Double-stranded RNA extraction

Double-stranded RNAs for analysis by gel electrophoresis and RNA sequencing were extracted and purified according to the previously described method (Fredericks et al. 2021a). To improve the efficiency of the extraction process for analysis by gel electrophoresis, several steps in the original protocol were altered.

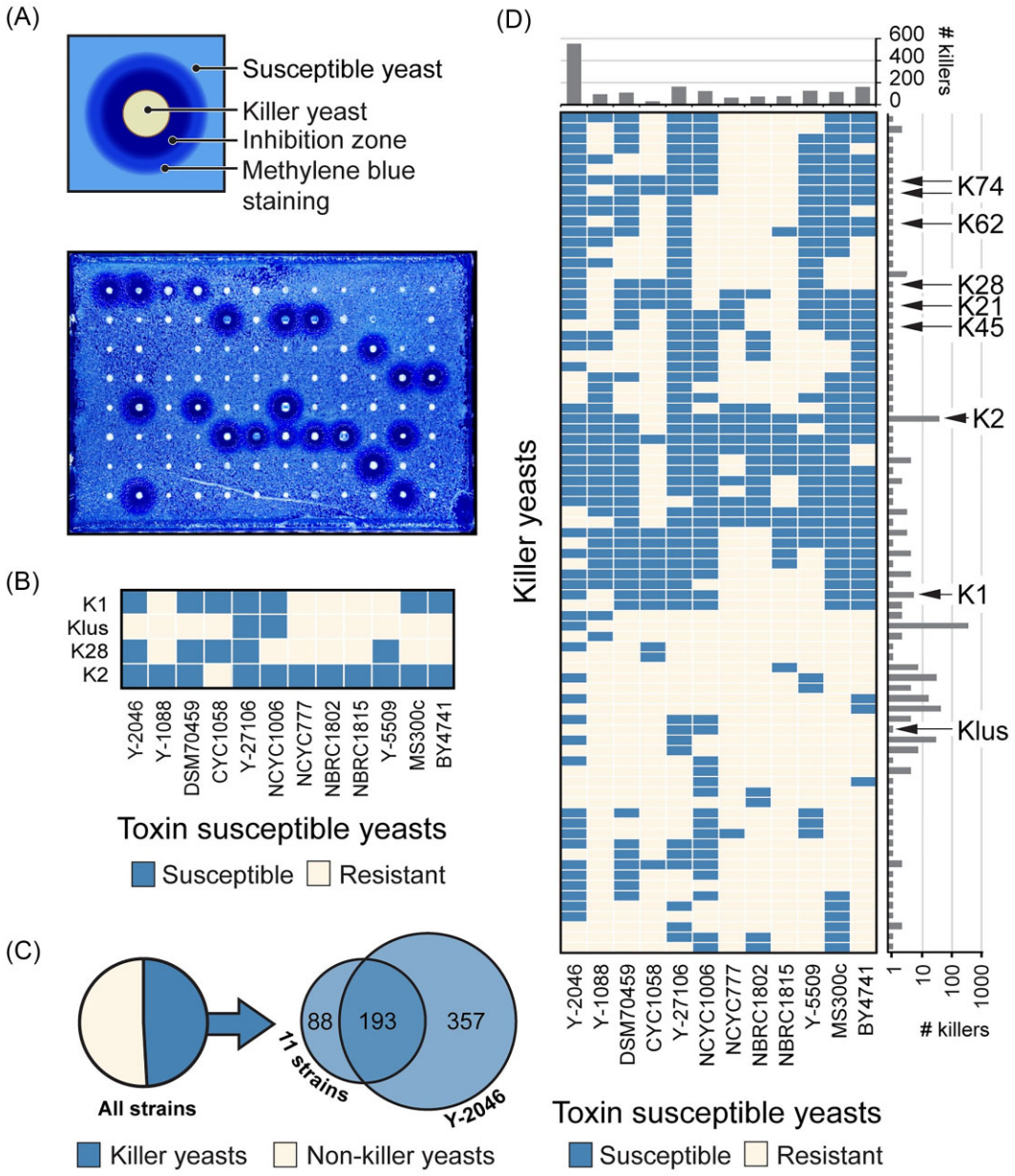


**Figure 1.** Killer toxin production is widespread in diverse strains of *S. cerevisiae*. (A) *top* Schematic representation of growth inhibition by killer yeasts on agar. *Bottom* Representative image of killer and non-killer yeasts with and without zones of growth inhibition, respectively. (B) Susceptibilities of 12 killer toxin susceptible lawn strains of yeast to four canonical killer toxins produced by *S. cerevisiae* YJM1307 (K1), DSM70459 (Klus), MS300c (K28), CYC1172 (K2). Blue boxes indicate observable growth inhibition by killer yeasts. (C) The proportion of *S. cerevisiae* killer yeasts identified by challenging 12 susceptible yeasts (all strains). Killer yeasts that inhibit strain Y-2046 relative to 11 other killer toxin-sensitive strains are also indicated. (D) A cluster diagram of killer yeasts that inhibit the growth of at least one susceptible lawn strain. Blue boxes indicate observable growth inhibition by killer yeasts, and yellow boxes indicate no growth inhibition. The vertical histogram represents the total number of yeasts that inhibited the growth of each killer toxin susceptible strain. Killer yeasts with identical spectrums of activity were collapsed into a single row, and the horizontal histogram ($log_{10}$ scale) represents the number of yeasts within each row.

Specifically, the volume of phenol-chloroform was reduced from 500 to 275 μL and approximately 2 mL of crude cell extract (from up to 50 mL of a 48 h yeast culture) was passed over a single cellulose spin column (Fig. S1). To increase the yield of dsRNA, the number of 1X STE buffer washes over the column was reduced to one. During the final dsRNA suspension step after dsRNA precipitation, a pipet tip was used to move 15–20 μL of water along the inner surface of the microcentrifuge tube to ensure a more complete recovery of dsRNAs.

## Double-stranded RNA sequencing

Purified dsRNAs were prepared for sequencing by adding 3′ poly(A) tails to purified dsRNAs and used as non-specific priming sites for cDNA synthesis, as described previously (Crabtree et al. 2019). Amplified cDNAs were prepared for Illumina sequencing using a modified Nextera library preparation method, and sequencing of cDNAs was performed with either the Micro v2 300 or Nano v2 500 cycle reagent kit on the Illumina MiSeq platform.

## Sequencing analysis

*De novo* contig assembly was performed after cleaning raw reads with fastp v0.20.0 and filtering out *Homo sapiens* and *Saccharomyces cerevisiae* read contaminants with BBMap's bbsplit algorithm (v.38.86) (Chen et al. 2018). SPAdes v3.14.1 produced *de novo* contigs from the remaining polished reads using default parameters and a read coverage cutoff of 5 (Bankevich et al. 2012). A local BLAST was performed using satellite sequences from the NCBI database, and significant hits were chosen for reference mapping. Reads were mapped using BWA, and read depth was determined with samtools v1.10 (using htslib 1.10) (Li et al. 2009). R v4.0.0, including packages ggplot2 and stringr, was used to graph assembly depth and contig coverage versus length. Consensus sequences were created by variant calling with bcftools v1.10.2 (using htslib 1.10.2). 5′ and 3′ terminal ends with no read coverage were truncated using Python3 v3.7.4.

## Identification of the S. cerevisiae chromosomal killer toxin genes, KHR1 and KHS1

A local BLASTx was performed on the *de novo* contigs and fully assembled chromosomes of yeast genomes using the Khs1 and Khr1 protein sequences available on NCBI (accessions EDN63163.1 and BAA00751.1, respectively). Hits were deemed positive with e-values smaller than 1e-30. Hits were validated and checked for truncations using custom Python3 and R scripts (see Github; https://github.com/amcrabtree/dsrna-survey)

## The cloning and ectopic expression of KHR1 and KHS1

The *KHS1* gene (*S. cerevisiae* YJM789 (protein SCY_1690)) (Frank and Wolfe 2009) was synthesized and cloned into a Gateway™ entry vector (pTwist ENTR) by Twist Bioscience to create plasmid pUI135, and *KHR1* was cloned directly from the genome of *S. cerevisiae* YJM1341. *KHR1* was amplified by PCR (Phusion polymerase; Thermo) using purified genomic DNA with the primers 5′-AAACGGCTATATATTTCGCGGTAGG-3′ and 5′-GCGCCAACAAGGCTATTTCG-3′ (PRUI3 and PRUI4, respectively). The *KHR1* PCR product was cloned into the Gateway™ entry vector pCR8 using Topo-TA cloning (Invitrogen) to create pAC007. *KHR1* and *KHS1* were then cloned into the multicopy plasmid pAG426-GAL-ccdB for galactose inducible expression to create plasmids pTwist-KHS and pUI136, respectively (Alberti et al. 2007). Ectopic expression was performed as described previously (Fredericks et al. 2021a).

# Results

## The prevalence and diversity of killer yeasts in *S. cerevisiae*

To identify killer yeasts in *S. cerevisiae*, 1270 strains from published collections representing the global diversity of the species were screened for killer toxin production (Strope et al. 2015, Ludlow et al. 2016, Peter et al. 2018) (Fig. 1A and Table S1). Four killer yeast strains known to produce different types of canonical killer toxins were used to select 12 strains of toxin-susceptible yeasts for this killer yeast screening approach (Fig. 1B). The 12 strains were selected based on their differing susceptibilities to killer toxins and their growth characteristics (i.e. reduced flocculation, robust growth at pH 4.6). Several killer yeasts were included in the 12 selected strains because of their intrinsic resistance to K2 (CYC1058), Klus (DSM704559), and K28 (MS300c) (Schmitt et al. 1996, Rodríguez-Cousiño et al. 2013, Fredericks et al. 2021a). After challenging the 12 susceptible lawns with 1 270 strains of *S. cerevisiae* using a high-throughput agar plate assay (Fig. 1A), it was found that 638 of the assayed strains (50.2%) were killer yeasts (Fig. 1C). The majority (66.6%) of killer strains could only inhibit a single susceptible lawn (Fig. S2). On average, the identified killer yeasts were able to inhibit the growth of 2.6 susceptible strains, and only one killer yeast strain inhibited all 12 challenged strains (ABT; Table S1). The MAT$\alpha$-haploid *S. cerevisiae* strain Y-2046 was inhibited by 550 killer yeasts, more than all other killer toxin-susceptible strains combined. 357 of these killer yeasts could only inhibit the growth of strain Y-2046 (Fig. 1C). Without including the hypersusceptible strain Y-2046, 22.1% of *S. cerevisiae* strains were identified as killer yeasts (Fig. 1C). The exclusion of Y-2046 decreased the percentage of killer strains that could only inhibit a single susceptible strain to 52.6% and increased the average number of strains inhibited by each killer yeast to 3.9 (Fig. S2). The susceptibility of Y-2046 did not appear to be due to the production of a-factor, as 64.2% of the killer yeasts that only inhibit the growth of Y-2046 are diploid and would not produce this mating pheromone (Fig. S3).

The activity of all 638 killer yeasts against 12 killer toxin-susceptible yeast strains can be represented by 72 unique groups, each with a distinct profile of antifungal activity (Fig. 1D). Canonical killer toxin types K1, K2, Klus, and K28 were used as reference points to compare to the 638 newly identified killer yeasts. Only 8.3% of killer yeasts had identical antifungal activities to known *S. cerevisiae* killer toxins. (Fig. 1D). Specifically, there were five killer yeasts with the same spectrum of activity as K1 and 48 strains the same as K2. The antifungal activities of four canonical *S. paradoxus* killer yeasts (K21, K45, K62, and K74) and two *S. cerevisiae* killer yeasts (Klus and K28) were unique compared to all killer yeasts identified. To assess killer phenotypes of identical yeast strains from different culture collections, duplicates of 79 strains were compared. 49.4% of these strains had identical spectrums of killer toxin activity across all 12 strains assayed (Fig. S4). Most differences between strains resulted from only a single mismatch, demonstrating the overall reproducibility of the killer phenotype between collections and the robustness of the assay to detect killer yeasts.

## The identification of polymorphic killer toxins with altered antifungal activities

To identify the killer toxins produced by killer yeasts with unique antifungal properties, dsRNAs were purified from ten strains and analyzed using a short-read sequencing pipeline. This analysis identified killer toxins with high nucleotide sequence identity (>92%) to the previously described toxins K1, K28, and Klus (Fig. S5 and Table S1). In particular, eight polymorphic K1 toxins were identified with non-synonymous mutations present in all functional domains ($\alpha, \beta, \gamma, \delta$) (Fig. S6). Relative to the previously characterized K1 toxin produced by BJH001 (Crabtree et al. 2019), K1 variants were found to kill more (YJM1290, YJM1307, YJM1287, and YJM1077) or fewer (NCYC190, YO1482, and YO1490) yeast strains. Where K1 toxins from different strains had the same mutations, antifungal activities were identical (Fig. S6; compare YO1621 and YO1622) or near identical (Fig. S6; compare YO1482 and YO1490). This demonstrated that mutations could modulate toxin activity against different yeasts, but whether this is due to changes in toxin potency, expression, or cell targeting remains to be determined.

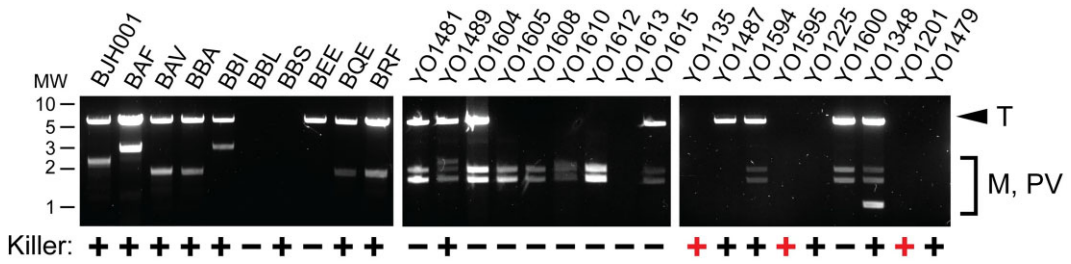


**Figure 2.** The diversity of dsRNAs extracted from *S. cerevisiae*. Agarose gel electrophoresis of representative dsRNAs extracted by cellulose chromatography from different strains of *S. cerevisiae* stained with ethidium bromide. (-) non-killer yeasts (+) killer yeasts (+) killer yeast that only inhibits the growth of Y-2046; T, high molecular weight dsRNAs indicative of totivirus dsRNAs; M, PV low molecular weight dsRNAs indicative of satellite dsRNAs and partitiviruses. Totivirus and satellite dsRNAs from the K1 killer yeast *S. cerevisiae* BJH001 were used as a positive control. MW; DNA molecular weight ladder. All dsRNA extraction data is presented in Fig. S5.

**Table 1.** The percentage of mycoviruses and satellite dsRNAs associated with killer and non-killer strains of *S. cerevisiae*. The types of dsRNAs identified in 134 killer and 39 non-killer yeasts are indented. Values in parentheses represent the number of yeast strains assayed.

| | Killer yeasts | Non-killer yeasts |
|---|---|---|
| **Without dsRNAs** | 63.1% (106) | 36.9% (62) |
| **With dsRNAs** | 77.5% (134) | 22.5% (39) |
| Totivirus only | 71.4% (40) | 28.6% (16) |
| Partitivirus only | 18.8% (3) | 81.2% (13) |
| Totivirus & satellite | 98.6% (73) | 1.4% (1) |
| Totivirus & partitivirus | 52.6% (10) | 47.4% (9) |
| Totivirus, partitivirus, & satellite | 100% (8) | 0% (0) |

## *S. cerevisiae* killer yeasts are biased in their environmental distribution.

The previous classification of killer yeasts by their ecological origin allowed the analysis of their prevalence in specific niches (Peter et al. 2018). For example, there were more killer yeast strains associated with human clinical and industrial processes (such as baking and brewing; 76.1%) than yeasts associated with natural habitats (21.6%). This association significantly differed from the proportion of non-killer yeasts from these niches (Chi-squared test; $P = 0.008$) (Table S2). Comparing the prevalence of killer yeasts in 22 different ecological niches, killer yeasts were significantly enriched in human clinical samples and European winemaking processes (Chi-squared test; $P < 0.002$ (Bonferroni corrected)). Conversely, non-killer yeast strains were significantly more abundant in samples from trees, sake, and non-clinical human samples (Chi-squared test; $P < 0.002$ (Bonferroni corrected)). Killer yeasts were enriched in the phylogenetic clades associated with winemaking (European wine and European wine subclade 3) mosaic yeast strains (Mosaic Beer and Mosaic region 3), and those of mixed phylogenetic origin. Non-killer yeasts were more prevalent in Sake, Alpechin, French Guiana, Ale beer, and French dairy clades (Chi-squared test; $P < 0.0016$ (Bonferroni corrected)) (Table S3). These results demonstrated that killer yeasts are biased in their distribution across natural and anthropic habitats.

## The prevalence of dsRNAs in *S. cerevisiae* and their correlation with killer toxin production

Killer toxin production in *Saccharomyces* yeasts has been frequently associated with mycovirus infection, enabling the stable maintenance of dsRNA satellites that encode killer toxins. To determine the prevalence of mycoviruses and satellites within *S. cerevisiae* killer yeasts, dsRNAs were extracted using cellulose chromatography from 341 randomly selected *S. cerevisiae* strains, including 240 killer yeasts and 101 non-killer yeasts. Gel electrophoresis was used to detect the dsRNAs and identify the different types of mycoviruses and satellites in each strain assayed by their electrophoretic mobility. Based on prior studies that have previously characterized mycoviruses (Wickner et al. 2013), monopartite totivirus genomes were identified as dsRNA species with an apparent molecular weight of ≈5 kbp, as judged by a dsDNA molecular weight standard (Fig. 2 and Fig. S7). Satellite dsRNAs were identified as single species of dsRNA ranging from 1-3 kbp that are always present with a totivirus (Fig. 2; e.g. BAF and BAV). Partitiviruses are bipartite mycoviruses that were identified as doublets of ≈2 and ≈1.5 kbp (Fig. 2; e.g. YO1605). In some cases, it was possible to observe strains with multiple dsRNAs representing co-infection by totiviruses and partitiviruses with satellite dsRNAs (Fig. 2; e.g. YO1489). Of all the *S. cerevisiae* strains assayed, 50.7% harbored dsRNAs (Table 1). Of these dsRNAs, 47.4% were totiviruses with satellite dsRNAs, and 52.6% were mycovirus dsRNAs without satellites. Partitiviruses were found in 12.6% of strains assayed, whereas totiviruses were present in 46.0%. Most killer yeasts contained dsRNAs (55.8%), and 60.5% of these dsRNAs were totiviruses with satellite dsRNAs. Conversely, only 38.6% of the 101 non-killer yeast strains assayed were found to contain dsRNAs. Of the non-killer yeasts assayed, 15.8% contained lone totiviruses, 12.6% lone partitiviruses and 8.9% had both types of mycoviruses (Table 1). Only one non-killer yeast strain was found to harbor a totivirus and satellite dsRNA (1% of non-killer yeasts assayed). As expected, killer toxin production was significantly correlated with the presence of satellite dsRNAs in killer yeast (Fisher's exact test, $P < 0.01$).

## Genome-encoded killer toxins in *S. cerevisiae*

159 killer yeasts did not contain satellite dsRNAs (106 strains without dsRNAs, 40 strains with only totiviruses, and 3 with only

**Table 2.** The percentage of killer yeast associated with the presence of *KHR1* and *KHS1*. Killer yeasts were defined as inhibiting one of 11 strains of killer toxin-sensitive yeasts or only *S. cerevisiae* Y-2046. Values in parentheses represent the number of killer yeasts assayed.

| | | only *KHR1* | only *KHS1* | no *KHR1*/*KHS1* |
|---|---|---|---|---|
| Killer yeast | 11 strains | 17.2% (44) | 60.2% (154) | 22.7% (58) |
| | Y-2046 only | 11.1% (14) | 77.8% (98) | 11.1% (14) |
| Non-killer yeast | 11 strains | 32.1% (144) | 17.0% (76) | 50.9% (228) |
| | Y-2046 only | 30.1% (174) | 22.8% (132) | 47.1% (272) |

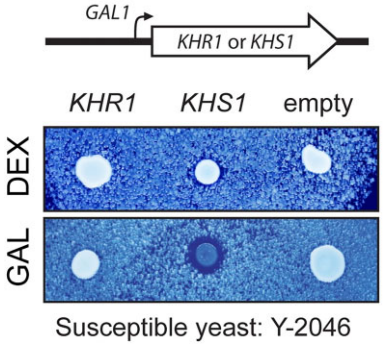


**Figure 3.** Ectopic expression of *KHS1* can inhibit the growth of *S. cerevisiae* Y-2046. The non-killer yeast *S. cerevisiae* BY4741 was transformed with a high-copy plasmid encoding either of the killer toxins *KHR1*, *KHS1*, or the vector alone. Both killer toxin genes were under the control of a galactose-inducible promoter. The transformed strains of *S. cerevisiae* were inoculated at high density on agar plates containing dextrose (DEX) or galactose (GAL) seeded with the hypersusceptible strain Y-2046.

partitiviruses), suggesting that the DNA genome of these strains encoded killer toxin genes (Table 1). To identify the possibility of genome-integrated killer toxins, the genomes of different strains of *S. cerevisiae* were analyzed using BLASTn for the presence of K1, K2, K28, Klus, K21, K62, K45, and K74 genes, but no significant hits were found. It has been previously shown that the DNA genome of *S. cerevisiae* encodes the killer toxin genes *KHR1* and *KHS1* that were able to inhibit the growth of *C. glabrata* (Goto et al. 1990, 1991). Although widely prevalent across the species, both genes are absent from the *S. cerevisiae* reference genome. The BLASTx analysis of publicly available genomes of *S. cerevisiae* enabled the identification of full-length *KHR1* and *KHS1* in 830 strains of *S. cerevisiae* (see methods). In 723 strains, either one or both of these genes were predicted to be non-functional due to premature stop codons, or indels, or were absent from the genome assemblies and sequence contigs. Comparing the number of killer and non-killer yeasts to the presence of only full-length *KHR1* or *KHS1* revealed that *KHS1* was significantly correlated with killer toxin production (Fisher's exact test, $P < 0.01$), compared to *KHR1* (Fisher's exact test, $p = 0.42$) (Table 2). The same correlation was also found when comparing the 357 killer yeasts that were only able to inhibit the growth of the hypersusceptible strain Y-2046 (Fisher's exact test, *KHR1* $p = 0.25$, *KHS1* $P < 0.01$) (Table 2). To confirm the susceptibility of *S. cerevisiae* Y-2046 to the killer toxins KHR and KHS, each toxin gene was cloned and conditionally expressed by the non-killer laboratory strain *S. cerevisiae* BY4741 that lacks both *KHR1* and *KHS1*. Only after transcriptional activation by galactose was *KHS1* able to inhibit the growth of *S. cerevisiae* Y-2046, whereas there was no inhibition upon the induction of *KHR1* (Fig. 3). These data suggest that *KHS1* is most likely responsible for the large numbers of *S. cerevisiae* killer yeast strains that inhibit the growth of *S. cerevisiae* Y-2046 but lack satellite dsRNAs.

## Discussion

Prior studies have broadly estimated killer yeast frequency, finding that killer yeasts represent between 2.5% and 78.8% of yeasts across diverse genera (Phillskirk and Young 1974, Stumm et al. 1977, Kandel and Stern 1979, Rosini 1983, Kitano et al. 1984, Heard and Fleet 1987, Starmer et al. 1987, Martini and Rosini 1989, Hidalgo and Flores 1994, Buzzini and Martini 2000, Carreiro et al. 2002, Baeza et al. 2008), and 1%–6% prevalence in *S. cerevisiae* (Pieczynska et al. 2013, Chang et al. 2015). A recent screen of the *S. cerevisiae* "100-genomes" collection for killer yeasts found that 69% of *S. cerevisiae* strains can inhibit the growth of *C. glabrata*, which appeared to be dependent on the presence of the genome-encoded killer toxin *KHS1* (Vijayraghavan et al. 2023). Our screening approach used 12 yeast strains susceptible to a range of killer toxins and found that 50.2% of 1270 tested *S. cerevisiae* strains are killer yeasts. The prior low detection rate of killer yeasts in some studies was likely due to a limited number and diversity of yeast strains used to identify killer toxin production, as killer toxin susceptibility varies considerably between different strains and species of yeasts. The higher number of killer yeasts identified in the current study was due to multiple strains of yeasts being used to detect killer toxin production and the inclusion of *S. cerevisiae* Y-2046, which was particularly sensitive to killer toxins, including KHS.

The high prevalence of satellite dsRNAs, *KHR1*, and *KHS1* means that many strains of *S. cerevisiae* can potentially produce multiple killer toxins. This complicates the interpretation of previous work that has identified killer toxins and inferred their spectrum of activity without considering the contribution of *KHR1* and *KHS1*. For example, several studies have determined that *C. glabrata* is particularly susceptible to *Saccharomyces* killer toxins, but without considering genomically encoded killer toxins (Middelbeek et al. 1980, Fredericks et al. 2021b). Using strains lacking *KHR1* or *KHS1* can provide a clearer picture of their contributions to the killer phenotype, such as using the laboratory strain S288c, which lacks both *KHS1* and *KHR1*, to express killer toxins. For example, a K1 killer yeast derived from S288c (strain BJH001) can inhibit the growth of *C. glabrata*, confirming the sensitivity of *C. glabrata* to K1 (Fredericks et al. 2021b). However, even in a detailed study of *KHS1* using the *S. cerevisiae* 100-genomes collection, the relationship between the *KHS1* gene and the inhibition of *C. glabrata* was still imperfect (Vijayraghavan et al. 2023). Moreover, the canonical *S. paradoxus* killer strain Q62.5 that produces K62 encodes a full-length *KHS1* but could not inhibit *C. glabrata* (Fredericks et al. 2021b). These discrepancies might be explained by polymorphisms that could alter the spectrum of *KHS1* antifungal activities or inactivate *KHS1*. Further investigation of the 14 killer yeast strains lacking *KHR1* and *KHS1* would enable a better explanation of their antifungal activities, such as the production of other toxins encoded by dsRNA satellites, linear DNA plasmids, or antimicrobial peptides (Klassen and Meinhardt 2002, Kemsawasd et al. 2015). Altogether, the diversity in dsRNA and DNA-encoded killer

toxins could explain the complex antifungal activities of *Saccharomyces* killer yeasts observed in the current study.

Awareness of the high prevalence of killer yeasts is critical to understanding their importance in many applications, including scientific studies using *S. cerevisiae* as a model organism and during industrial fermentation. For example, experiments that co-culture diverse yeasts (e.g. genome-wide association studies and quantitative trait locus mapping) could be susceptible to bias due to the lethal effects of killer toxins, as killer yeasts can outcompete and invade non-killer yeast populations (Pintar and Starmer 2003, Greig and Travisano 2008, McBride et al. 2008, Wloch-Salamon et al. 2008). Contamination by killer yeasts can also result in industrial spoilage, as low concentrations of killer toxins can trigger the death of sensitive cells (Maule and Thomas 1973, Reiter et al. 2005, Sheppard and Dikicioglu 2019). However, the current study shows that killer toxin production is significantly more prevalent in winemaking strains, and it has been shown that K2 is highly active at pH values relevant for winemaking (Rodríguez-Cousiño et al. 2011, Ullivarri et al. 2014). In some studies, winemaking killer yeasts can prevent the contamination of other strains of *S. cerevisiae* but are limited in their abilities to inhibit the growth of contaminating non-*Saccharomyces* yeasts (Heard and Fleet 1987, Hidalgo and Flores 1994). Other investigations have shown conflicting results, with killer yeasts being unimportant or even detrimental to winemaking (such as (Jacobs and Vuuren 1991, Gutiérrez et al. 2001, Zagorc et al. 2001, Vuuren and Wingfield 2017)). This study shows that killer yeasts are less prevalent in other human fermentations (e.g. beer, sake, etc.), so killer toxins may be particularly advantageous for winemaking yeasts. However, the conflicting evidence regarding their relevance during winemaking could mean that killer toxin production is perhaps more important for persistence in vineyards or insect vectoring (Becher et al. 2012, Stefanini et al. 2012, Stefanini 2018, Buser et al. 2021).

The close association between killer toxin production and infection with totiviruses and satellite dsRNAs has likely biased prior estimates of mycovirus prevalence because killer toxin production is used as a proxy for viral infection. The current survey of mycoviruses in non-killer yeasts found that 37.6% of strains contain only mycoviruses, with only 1% of non-killer yeasts having satellite dsRNAs. This agrees with a similar screen for dsRNAs in the 100 genomes collection as observed by agarose gel electrophoresis (Vijayraghavan et al. 2023). As the current study included many of the strains assayed by Vijayraghavan et al., we could compare results from dsRNA extractions for 19 strains. Overall, similar results were observed, with the main difference being increased sensitivity to detect satellite dsRNAs using cellulose chromatography in 5 strains. In addition to gel electrophoresis, Vijayraghavan et al. employed a PCR-based approach to detect totiviruses in *S. cerevisiae*. They found that 30% and 100% of strains harbored L-A and L-BC, respectively. However, these data only estimate the prevalence of mycoviruses in *S. cerevisiae*, as mycoviruses can be biased to strains from specific ecological niches, such as partitiviruses (Taggart et al. 2023). Additional biases are associated with PCR-based mycovirus detection methods being limited to known viruses. The unbiased sequencing of dsRNAs extracted from *S. cerevisiae* identified contigs related to many different classes of viruses and was responsible for the recent discovery of partitiviruses in *S. cerevisiae* (Taggart et al. 2023). More comprehensive unbiased screens of *S. cerevisiae* mycoviruses will likely uncover their true diversity and prevalence in the species. This is important because there are examples of mycovirus-dependent phenotypes in *S. cerevisiae*, such as increased copper tolerance, increased sporulation, slow growth, and cell death (Dihanich et al. 1989, Liu and Dieckmann 1989, Edwards et al. 2014, Cook et al. 2022, Vijayraghavan et al. 2022, 2023). Moreover, loss of specific host nucleases and other antiviral proteins increased L-A copy number and, in some cases, caused a reduction in cell viability (Toh-E et al. 1978, Ridley et al. 1984, Liu and Dieckmann 1989, Rowley et al. 2016, Gao et al. 2019, Chau et al. 2023). This highlights the broader importance of understanding how mycoviruses can affect the physiology of *S. cerevisiae*. As with killer toxin production, mycoviruses could also impact industrial fermentation and the academic study of fundamental biological processes.

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## Supplementary data

Supplementary data is available at *FEMSYR Journal* online.

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