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LEHIGH UNIVERSITY

Quantifying Recombination Rate of Yeast Killer Virus

A Thesis Submitted in Partial Fulfillment of the
Requirements for the Eckardt Scholars Program

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Abstract

The yeast *Saccharomyces cerevisiae* is host to a selfish intercellular “Killer” virus. The yeast Killer virus is a double stranded RNA (dsRNA) that encodes a Killer toxin and confers immunity to the toxin. This virus was first described over 40 years ago, however, we still have a limited understanding of many aspects of the viral system, including frequency of viral recombination. Here we perform pairwise crosses between yeast strains containing known killer virus mutants that are unable to produce functional toxin. Successful mating events are then screened for killing ability to identify instances of recombination, which is denoted by reconstitution of killing phenotype. We have been unable to identify any instances of recombination in over 16 unique crosses where functional recombinants could have been generated. Sequencing reveals that both Killer virus variants are stably maintained as a heteroplasmic viral population. Our results suggest that killer virus has a low recombination frequency, which may extend to other encapsulated cytoplasmic RNA viruses as well.

Introduction

Killer yeast strains secrete protein toxins that are capable of killing surrounding non-killer yeast cells, yet are immune to their own toxin as well as those produced by other yeast strains infected with the same Killer strain (Ramírez, 2015; Magliani, Conti, Gerloni, Bertolotti, & Polonelli, 1997; Nieves Rodríguez-Cousino, 2017). In the wine and brewery yeast, *Saccharomyces cerevisiae*, the Killer phenotype is caused by infection with a cytoplasmic dsRNA virus that is a member of the *Totiviridae* family (Manfred J. Schmitt F. B., 2006; Bruenn, 1980; Nieves Rodríguez-Cousino, 2017). Yeast Killer virus, also known as M, is 1.6 to 2.4 kb in size (Manuel Ramírez, 2017) and is capable of infecting various species of yeast (Ramírez, 2015; Manfred J. Schmitt, 2002). It is comprised of a single open reading frame (ORF) that encodes a preprotoxin protein, which is processed by the host machinery into a mature toxin and secreted out of the cell (Schmitt, 2002). Killer virus is a satellite to a separate dsRNA helper virus, known as L-A, which is responsible for replication and encapsidation of the virus-like particles, or VLP's (Manfred J. Schmitt F. B., 2006; Nieves Rodríguez-Cousino, 2017a). Infection of yeast cells with L-A helper virus is symptomless and is not associated with any observable phenotype (Magliani, Conti, Gerloni, Bertolotti, & Polonelli, 1997; Schmitt F. B., 2002).

L-A viral genome is 4.6 Kb and contains two overlapping ORF's (Nieves Rodríguez-Cousino, 2017b). The first ORF encodes the structural protein of the capsid (Gag), which is required for encapsidation and viral particle structure (Manfred J. Schmitt F. B., 2006). Viral RNA polymerase (Pol) encoded by the second ORF and is translated as a fusion protein (Gag-

Pol) of the two overlapping ORF's by a -1 ribosomal frame shifting (Nieves Rodríguez-Cousino, 2017a; Dinman, Icho, & Wickner, 1991). Replication of L-A dsRNA occurs first through the expression of the Gag-Pol fusion protein, which synthesizes new dsRNA while the Gag domain allows for free Gag monomers to polymerize on it and form the capsid (Fujimura, Esteban, & Wickner, 1986; Fujimura T. a., 663-671). L-A virus can support the replication and encapsidation of the M Killer virus, with the difference that two dsRNA M copies are contained in each VLP as opposed to one copy for L-A (Magliani, Conti, Gerloni, Bertolotti, & Polonelli, 1997). Both Killer and Helper virus are incapable of horizontal transmission, instead relying on vertical transmission during yeast mating events and subsequent sporulation (Manfred J. Schmitt F. B., 2002)

The first killer yeast was discovered in a strain of *S. cerevisiae*, which was isolated as a brewery contaminant (Bevan & Makower, 1963). After more than 50 years of research, much of the Killer system remains unknown, though there have been some interesting discussions about potential applications (Special Issue "Yeast Killer Toxins", 2017). We are interested in crossing strains together to understand mutational variants of Killer virus. A previous experiment included crossing two purported Killer-cured, or K^0 strains was performed with the intention of identifying the factor that led to curing. Contrary to the expected results of isolated progeny displaying a non-killing (or K^-) phenotype, all resulting spores from the crosses had the wild-type killer phenotype (K^+). These results, combined with the indication that the K^0 strains were in fact K^- mutants, suggested that a recombination event had occurred between two killer variants, producing a wild-type Killer variant.

It has already been suggested that Killer virus is capable of undergoing viral recombination (Manuel Ramírez, 2017). There is high sequence identity shared between different M viruses as well as RNA from certain plant species in the 5' and 3'-extra sequences (Manuel Ramírez, 2017). This suggests that viral recombination events can occur along with recombination with the yeast genome and the organism in which the host cell resides. It is unclear what the mechanism of recombination is for dsRNA viruses (Etienne Simon-Loriere, 2011). In this paper, we have devised a method in which we can approximate the frequency of recombination for the M1 yeast Killer virus. By crossing yeast strains containing viral mutants, we observed the frequency of reconstitution of wild-type killer phenotype and showed initial evidence of possible intracellular viral competition.

Materials and Methods

Isolation of Killer Virus Mutants

We used the *MATa* yeast strain, yGIL 432 (*MATa*, *ade2-1*, *CAN1*, *his3-11*, *leu2-3,112*, *trp1-1*, *URA3*, *bar1Δ::ADE2*, *hmlαΔ::LEU2*, *GPA1::NatMX*, *ura3Δ::PFUS1-yEVENUS*) and subjected it to various growth conditions. 2ml liquid cultures of yGIL 432 were incubated overnight at 30 degrees Celsius, then 100 μl of a 10⁻⁵ dilution was spread onto plates. They were then incubated for 48 hours at experiment-dependent temperatures, of which 96 colonies were picked from the plate and inoculated in 125 ul YPD (Yeast Extract, Peptone, Dextrose) on a 96-well plate. After incubation for 24 hours at 30° C, we performed a high-throughput killer assay to screen for killing ability of different colonies (see killer assay section in methods). K⁺ strains

were expected to exhibit a zone of clearing around them. K^- or K^0 strains are expected to show no zone of clearing. If there are any K^- spots, 5 μ l of the culture for that colony from the 96-well plate is streaked out onto a YPD plate and grown up for 48 hours at 30°C. A single colony was picked from the plate to create a 5 ml YPD culture, which was incubated overnight at 30°C. One milliliter was subsequently used to run a basic killer assay in order to reconfirm killing phenotype (see killer assay section in methods). Known Killer-containing and Killer-cured strains, W303 and S288C, were used as positive and negative controls. If the assay confirms that the test strain is K^- , 200 μ l of that single colony is mixed with glycerol and added to a 96-well plate and frozen down. The following factors were considered for environmental perturbations: temperature, presence/absence of 5-fluoroorotic acid (5FOA), and pH.

We additionally wanted to isolate killer mutants in the $MAT\alpha$ mating background so as to cross them with isolated $MATa$ mutants. We focused on moving killer mutants in $MATa$ background from my killer library to $MAT\alpha$ background. This was achieved by mating $MATa$ Killer variant strains with a K^0 $MAT\alpha$ strain. For this, we used yGIL 1253 ($MATa$, *ade2-1*, *CAN1*, *his3-11,15* *leu2-3,112*, *trp1-1*, *URA3*, *bar1 Δ ::ADE2*, *hml α Δ ::LEU2*, *GPA1::NatMX*, *ura3 Δ ::PFUS1-yEVENUS*), which has a KanMX cassette replacing the NatMX cassette at the GPA1 locus.

We decided to move four M1 Killer variants from the $MATa$ background into a $MAT\alpha$ yeast strain. The Killer variants chosen for this were spontaneous mutants named A06, A08, A09, as well as yGIL 432 as a control. Single colonies from A06, A08, A09, and 432 as well as the recipient yGIL1253, a G418-resistant strain, were isolated. A single colony from each donor strain was smeared onto a new YPD plate and patched with a single colony from yGIL 1253-KanR to mate them. The patches were then replica plated onto YPD+G418/ClonNat plates to

select for diploids. We streaked diploids to singles and made monoclonal YPD cultures for all four crosses. 10 microliters of culture was added to SPO++ sporulation media and incubated for 5 days to sporulate cells. Tetrad dissections were performed on tetrads generated from all four crosses on a YPD plate using a dissection microscope. All four-spore viable tetrads were inoculated in 200 μ l YPD on a 96-well plate.

To assay killing phenotype, tetrads were pinned onto buffered methylene blue (BMB) plates with yGIL1097 hypersensitive lawn (see “basic killing phenotype Killer assay). Tetrads were also pinned onto YPD+G418 and YPD+ClonNat plates to screen for resistance cassette segregation. As previously isolated *MATa* Killer variants already contain a NatMX drug resistance cassette, we selected for tetrads with the KanMX cassette. We used dual drug markers to screen for successful diploids in our following Killer mutant crosses. To isolate *MAT α* tetrads for crosses with our *MATa* Killer variants, we also pinned tetrads to YPD plates containing a lawn of yGIL354 (*met6 Δ ::KanMX*) *MATa* and yGIL353 (*met6 Δ ::KanMX*) *MAT α* mating-type-tester strains. After incubation for 48 hours at 30 degrees Celsius, we replica plated to SD minimal nutrient plates. Only tetrads that mate with the tester strain are able to survive on the plate. From these assays, we isolated *K⁻*, *MAT α* , KanMX tetrads and created 2ml YPD cultures. After plating 10 μ l onto a YPD agar plate, plates were incubated for 48 hours at 30° C and two isogenic colonies from each isolated tetrad were added to our strain library.

Pairwise Killer mutant crosses

Crosses consisted of first isolating monoclonal cultures from library stocks by streaking to a YPD plate and picking single colonies for 2ml YPD cultures. We performed a total of two

crosses in this experiment. See table 2 for a list of all strains used in crosses. The first round of crosses consisted of individually plating 5 μ l of each strain to be crossed onto a YPD plate in a grid-like fashion. This was done for all *MATa* and *MAT α* strains being crossed. Once patches grew on YPD plate, they were replica plated over to YPD+G418/ClonNat to select for diploids. Diploid isolates were then phenotyped for Killer ability (see “phenotyping killing ability of Killer mutant cross isolates”).

The second cross was performed using a different method. 2 ml monoclonal YPD cultures of the strains to be crossed were created again and incubated for 24 hours at 30°C. 50 μ l of each *MATa* and *MAT α* strain to be crossed was pipetted into a well on a 96-well plate. The wells were thoroughly mixed and 10 μ l of each cross was spotted onto YPD plates. After 48 hours at 30°C, the spots were then replica-plated to YPD + G418/ClonNat dishes to select for diploids. Diploid isolates were then screened for Killer phenotype as described in the previous section (see “Phenotyping killing ability of Killer mutant cross isolates”).

Phenotyping killing ability of Killer mutant cross isolates

Diploid progeny from Killer mutant crosses were first replica plated all from their double-drug plates to BMB plates with hypersensitive lawn to screen killing ability of diploid spots (see “basic Killer phenotype assay”). The next way of determining Killer phenotype was to swab diploid colonies from each cross and inoculate in 2 ml liquid YPD. After incubation for 24 hours at 30°C, 10 μ l of diploid culture was mixed with 10 μ l of yGIL 1097 hypersensitive strain and 10 μ l of the resulting mixture spotted onto BMB one-well plates. Speckling in spots would

suggest that there is active killer killing off hypersensitive cells and was thought to be more sensitive individual recombination events than in the previous method. The third method was to swab diploid colonies from all crosses and incubate in 2ml liquid YPD for 24 hours at 30 °C. Afterwards, 5 µl of each culture was struck onto YPD agar media and incubated for 24 hours at 30°C. Individual colonies were then picked, which should each correspond to a single mating event, and inoculated in 200 µl YPD on a 96-well plate. A high-throughput killer assay was then performed to screen all diploids for killing ability (see “high-throughput Killer phenotype assay”).

Reverse-transcription PCR of Killer mutant library

Two milliliter YPD overnight cultures were set up and incubated for 24 hours at 30°C. One milliliter of saturated culture was then resuspended in 200 µl of lysis buffer and approximately 0.2 grams of glass beads added to each sample. 200 µl of Phenol:Chloroform was then added. After vortexing for 10 minutes, samples were spun down for 5 minutes at top speed and 150 µl of the top organic layer added to 300 µl 100% ethanol. RNA was precipitated in a -20°C freezer for at least 30 minutes and subsequently spun down for 10 minutes at top speed. The ethanol was aspirated off and 1ml of 70% ethanol added to each sample. After vortexing and spinning down for 2 minutes at top speed, ethanol was aspirated off and the pellet resuspended in 50 µl of water. RNA was stored at -20°C or immediately used for synthesis of cDNA.

To synthesize viral cDNA, the Protoscript II First Strand cDNA Synthesis Kit was used from New England Biolabs. 0.5 µl of isolated RNA was added to 2.5 µl of water and 1.0 µl of a

provided random primer mix. Samples were then vortexed and quickly spun down to collect liquid at the bottom, then incubated for 5 minutes at 65°C. After letting the mix cool for 1 minute, 5.0 µl of 2X Protoscript II reaction mix and 1.0 µl of 10X Protoscript II enzyme mix were added. Samples were vortexed and quickly spun down again before leaving them to incubate for 1 hour at 42°C. Once 1 hour passed, samples were incubated for 5 minutes at 80°C and subsequently cooled at room temperature for another 5 minutes. cDNA was stored at -20°C or immediately used for PCR amplification of the M1 Killer or L-A-1 ORF.

PCR amplification of the Killer/ Helper ORF was performed using Q5 high-fidelity DNA polymerase and supplied Q5 buffer from New England Biolabs. 0.5 µl of viral cDNA was used in a 12.75 µl PCR reaction for 35 cycles (98°C for 30 sec, 98°C for 10 sec, 67°C for 30 sec, 72°C for 45 sec, 72°C for 2 min for final elongation, 10°C hold). To screen for the presence of M1 Killer virus, primers M1 F1 (5'-TTGGCTATTACAGCGTGCCA-3') and M1 R6 (5'-ATAGCCCGGTGCTCTGTAGG-3') were used. To amplify the entire ORF of the K1 ORF in M1 virus, primers M1 F5 (5'-ATGACGAAGCCAACCCAAGT-3') and M1 R6 were used. To screen for the presence of L-A-1 Helper virus, primers LA F2 (5'-ATCSGGTGATGCAGCGTTGA-3') AND LA R2 (5'-CGGCACCCTTACGGAGATAC-3') were used. PCR reactions were run on a 1% agarose + Ethidium Bromide gel to visualize results. For Sanger sequencing, PCR reactions were cleaned up using a Qiagen Qiaquick PCR Purification Kit. 12.5 µl of PCR product was added to 2.5 µl of either M1 F5 forward primer or M1 R6 reverse primer.

Analysis of Allele frequency of Killer variants in diploid isolates

Isolated cDNA of diploid isolates from the P47S x I292M Killer mutant crosses was Sanger-sequenced using primers M1 F5 and M1 R6. Chromatogram data was visualized using 4Peaks and SnapGene. To calculate the allele frequency for the different variants, peaks of the recorded nucleotides were measured at position 152 for P47S and position 889 for I292M. Peak height for nucleotides C and T were recorded for P47S as well as A and G for I292M. The peak of the mutant allele was then divided by the sum of the wildtype and mutant alleles to provide allelic frequency.

Basic Killer phenotype assay

To perform a basic screen for the killer phenotype of a yeast strain, the subject strain was inoculated in 2ml of YPD and incubated at 30 degrees Celsius for 24 hours. Overnight YPD cultures of K⁺ yGIL 432 and K⁻ yGIL 439 (*MATa*, *ade2-1*, *CAN1*, *his3-11*, *leu2-3,112*, *trp1-1*, *URA3*, *bar1Δ::ADE2*, *hmlαΔ::LEU2*, *GPA1::NatMX*, *ura3Δ::PFUS1-yEVENUS*) were additionally created to serve as positive and negative controls, respectively. One milliliter of culture was spun down at 15,000 RPM and the supernatant removed to concentrate cells. Ten microliters of distilled H₂O was then added to resuspend the solution and 100 μl of yGIL 1097 (*MATα*, *ade2-1*, *CAN1*, *his3-11,15*, *leu2-3,112*, *trp1-1*, *bar1Δ::ADE2*, *hmlαΔ::LEU2*, *GPA1::NatMX*, *ura3Δ::PFUS1-yEVENUS*), a K^o strain hypersensitive to Killer toxin, was spread out on a BMB plate. 10 μl of the concentrated subject strain and control solutions were then spotted and the plate left to incubate for 48 hours at room temperature. Killer phenotype was assigned based on the zone of clearing created by the test strain. A positive control will create a large zone of clearing in the hypersensitive lawn while the negative control will create no such zone.

High-throughput Killer phenotype assay

For screening of Killer phenotype in multiple yeast populations, we utilized a Biomek FX automated workstation to streamline the process. Colonies from a candidate yeast strain were isolated on YPD agar plates and inoculated in 200 μ l liquid YPD in a 96-well plate. After growth at 30°C for 24 hours, the supernatant was removed to leave 10 μ l. On a 1-well BMB plate, 125 μ l of hypersensitive yGIL 1097 was spread-plated. 2 μ l of the concentrated solution was then spotted on the BMB plate and incubated at room temperature for 48 hours. Killer phenotype was scored the same way as previously described (see “basic Killer phenotype assay”).

Media plates used

Buffered methylene blue plates were created by adding 10ml phosphate-citrate buffer (pH = 3.1) and 10ml of 0.3% methylene blue solution to 500 ml of melted YPD agar. SD+5FOA plates were made with a 5FOA concentration of 1X. For YPD+MOPS plates, 15 ml of 1M MOPS-OH buffer was added to 500ml melted YPD agar (Rokes, Unpublished).

Results

Lower temperature, plus 5FOA may lead to increased rates of M1 Killer mutants

To isolate mutants of M1 Killer virus for crosses between viral variants, we grew the strain yGIL 432 in the presence or absence of 5FOA as well as in various temperature and pH conditions. Colonies were grown on YPD, YPD+MOPS, and SD+5FOA plates with pH values of 4.5, 5.5, 6.0, and 6.5, respectively. These plates were incubated at either 23°C, 30°C, or 37°C and individual colonies were screened for Killer phenotype. In total, 17 mutants were isolated across all growth conditions (see table 3 for the full M1 mutant library). As the number of colonies screened from each growth condition have not been fully counted yet, we are unable

to calculate the frequency of the K^- for each condition at this time. Of all conditions screened, colonies grown at 23°C resulted in the largest total amount of Killer mutants isolated, at 9 colonies total. 23°C is the purported temperature at which M1 virus is most active, and previous studies have found that growth at higher temperatures leads to increased loss of Killer activity (Wickner, 1974). When all three factors of temperature, pH and 5FOA presence are included, however, colonies grown on SD+5FOA plates (pH 6.0) at 30°C had the greatest number of Killer mutants isolated, with 4 mutants. Again, elevated temperatures and high pH have been suggested to lead to increased loss of Killer activity (Wickner, 1974). 5FOA, an analog of uracil, has also been indicated to lead to increase rates of Killer curing (Magliani, Conti, Gerloni, Bertolotti, & Polonelli, 1997).

Loss of Killer virus is favored over inactivated Killer variants

We performed a forward genetic screen to isolate Killer mutants from varied growth conditions, of which 17 mutants were isolated. RT-PCR results of Killer mutants A01-A08 and A9 suggested that killer variants A01-A04 were k^- while variants A05-A08 and A9 were K^0 (see Figure 3 for gel results of Killer mutant RT-PCR). Using primers M1 F1 and M1 R6 to amplify a part of the M1 ORF, only variants A01 to A04 contained an approximately 500bp band. However, when using M1 F5 and M1 R6 to amplify the entire M1 ORF, only A01 contained an approximately 1.0Kb DNA band. This suggests that something in the upstream region of the M1 ORF is inhibiting replication of the entire sequence. It could be a mutation in promoter, for instance. When looking at the results of L-A Helper virus RT-PCR, Killer variants A01-A08 and A9 all display an approximately 900bp band, which is consistent with the size of the sequence amplified by the primers LA-F2 and LA-R2. However, the negative control lane also displayed a

900bp band; it is thought that this was caused by contamination from the positive control lane. The validity of the results may be called into question, but when taken in conjunction with the Killer RT-PCR results, A05 to A08 and A9 all appear to have lost M1 Killer virus while still retaining L-A-1 Helper virus.

Reconstitution of wild-type Killer was not observed

Two crosses were performed in this experiment. In the first cross, yeast strains containing viral mutants A06, A08, and A9 in both mating type backgrounds were crossed with each other. 96 diploid colonies from all collective crosses were screened for Killer phenotype. As A06, A08, and A9 are all K^0 however, a K^+ phenotype was not expected to be observed. In the second cross performed, we crossed the characterized Killer variants, P47S; D106G; I292M; and 119FS in both *MATa* and *MAT α* backgrounds. Progeny from crosses between four different mutant Killer strains were assayed for reconstitution of wild-type killing ability as evidence of a viral recombination event. 126 colonies were picked in total across all possible crosses and killing ability was assayed for killing phenotype as described in table 1, with each colony representing a separate mating event. All screened colonies displayed the k^- phenotype: there was no observance of reconstitution of the k^+ phenotype.

We repeated the cross between our two Killer variants containing the furthest mutations in the viral genome, P47S and I292M, so as to decrease genetic linkage and maximize our likelihood of recombination. From the YPD +G418/ClonNat plate, progeny were struck to singles and 96 colonies picket and inoculated in 200 μ l YPD. The 96 cultures were subsequently assayed for killing ability. Similar to our previous results, all colonies were k^- and there was no observance

of a k^+ colony. This is in contrast to the results of our crosses performed in previous experiments.

Allele frequency of Killer variants in diploid cells

To characterize the status of the killer mutants in the diploids produced from our crosses, we performed RT-PCR for five separate colonies isolated from our second P47S x I292M cross. Isolated viral cDNA was PCR amplified to isolate the complete M1 ORF and Sanger sequenced to check for the presence of both variants in our diploids. Figure 4 summarizes the sequencing results of these isolates. We are able to detect both P47S and I292M variants in four of the variants that were sequenced. Isolate 5 yielded clear sequencing results using the M1 F5 forward primer, but not with the M1 R6 reverse primer. The chromatogram data appeared to suggest the presence of something else apart from our sample of interest. Though both Killer variants are present in the background of isolated diploids from the P47S x I292M cross, there appears to be a disproportionate representation of I292M in all isolates. On average, the allele frequency of I292M present in them was 0.880, as opposed to 0.112 for P47S (unshown data).

Discussion

We have devised a method in which viral recombination frequency of yeast Killer virus can be approximated. Using a forward genetic screen, we isolated a variety of Killer variants and, after mating with a K^0 , KanMX strain, constructed a library of mutants in both *MAT α* and *MAT α* mating types. The constructs were then crossed with each other in a series of pairwise matings and their diploid progeny screened for Killer phenotype. The three Killer variants to be

used in our crosses, A06; A08; and A9, all did not contain M1 Killer virus upon RT-PCR and could not be used. Instead, four previously characterized Killer mutants, P47S; D106G; I292M; and 119FS, were crossed and 126 colonies screened for wild-type killing ability. Refer to table 1 for all crosses performed.

Our results indicate that if recombination is occurring between Killer viruses, it is rare: 0 out of 126 colonies screened showed a zone of clearing, suggesting that WT recombinant virus was not present. This is not enough to give an approximation of viral recombination frequency, as these results are only an indication of the penetrance of the non-killing phenotype. The possibility exists that recombination may be occurring, but not resulting in reconstitution of wild-type killer. Other portions of the genome may recombine, but we only expect to observe a change in phenotype when the two characterized mutations in both mutants recombine to produce a copy of Killer with no mutations. We also performed a cross a second time between two variants with the furthest genomic distance between them to maximize the likelihood of recombination. The 96 isolated diploids from this cross were phenotyped for killing ability and all displayed the k- phenotype again. This further suggests that recombination rate is increasingly low.

It is also possible that a wild-type copy of Killer virus can be outcompeted by another Killer variant. It has been suggested that separate viral variants can undergo intracellular competition, leading to the fixation of one variant in a population (Rokes, Unpublished). Two full-length Killer mutants as well as one mutant containing an intra-viral deletion were moved into the same background as wild-type Killer and the heteroplasmic strains propagated for 1000 yeast generations. Killing and resistance phenotype assays showed that populations gradually

lost killing ability over time, indicating that the WT copy is being outcompeted. There is some fitness benefit associated with the mutant strains, allowing them to fix in the population (Rokes, Unpublished); this phenomenon may also be applied to the crosses we performed in this experiment. Though previous experiments have not involved crosses between different killer mutants (Rokes, Unpublished), there is still the potential that one mutant may be outcompeted by the other and driven to low intracellular frequencies, thus hindering chances of recombination between the two viral variants. Looking further into intracellular competition between copies of M1 Killer virus with different mutations can help elucidate the Killer system as well as help us potentially mitigate this effect.

The Killer virus system still remains to be fully understood after more than 50 years of study (Special Issue "Yeast Killer Toxins", 2017). Our experiments can help clarify the relationship that exists between Killer virus and the host yeast cell for other evolutionary experiments that involve infected yeast strains. The association between specific M viruses and L-A Helper viruses has already been suggested to be resultant from coevolution (Nieves Rodríguez-Cousiño, 2017; Nieves Rodríguez-Cousino, 2017). In addition, Killer virus could be involved in a parasite-host coevolutionary relationship with the host yeast cell (A. Carolin Frank, 2009). Viral recombination can increase variation of yeast Killer virus in a population and potentially lead to reconstitution of WT killing ability in Killer variants, while laying the groundwork for further studies concerning viral competition.

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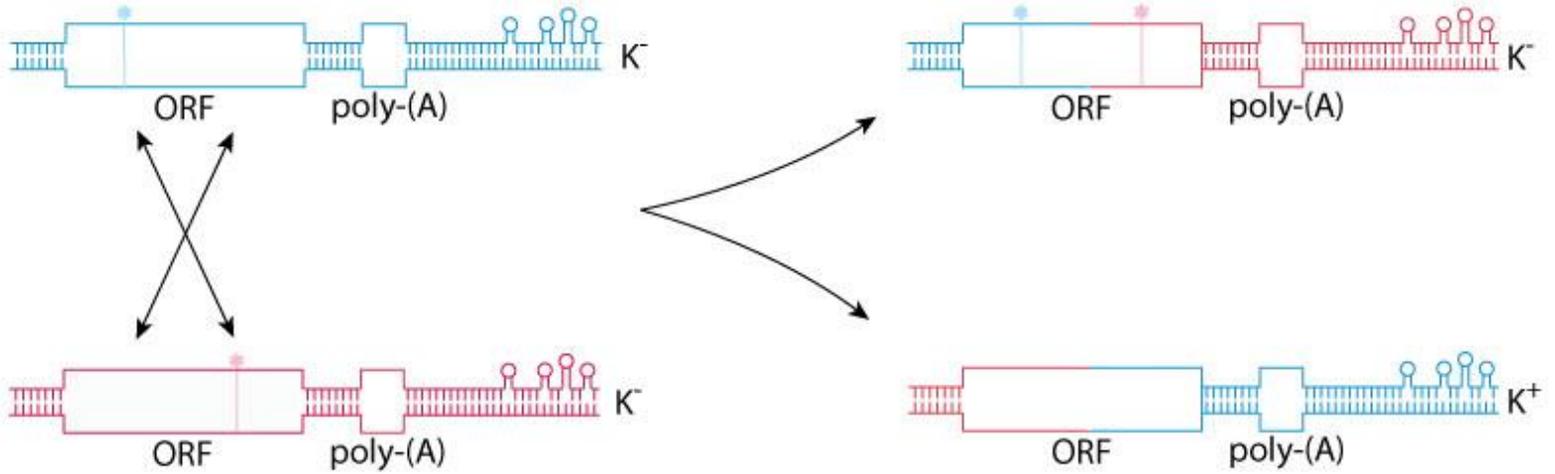


Figure 1. Viral recombination of two yeast Killer virus mutants. In the same cell background, two different Killer mutant variants will exchange genetic material at some frequency. One of the possible recombination outcomes is creation of a *wt* copy of Killer virus, reconstituting K⁺ phenotype.

TABLE 1. Crosses performed between characterized Killer virus variants

Killer Mutant Crosses	# Isolates Screened	# Killing Isolates	# Non-Killing Isolates
P47S x D106G	34	0	34
D106G x I292M	34	0	34
P47S x I292M	34	0	34
D106G x I19FS	24	0	24
Total	126	0	126

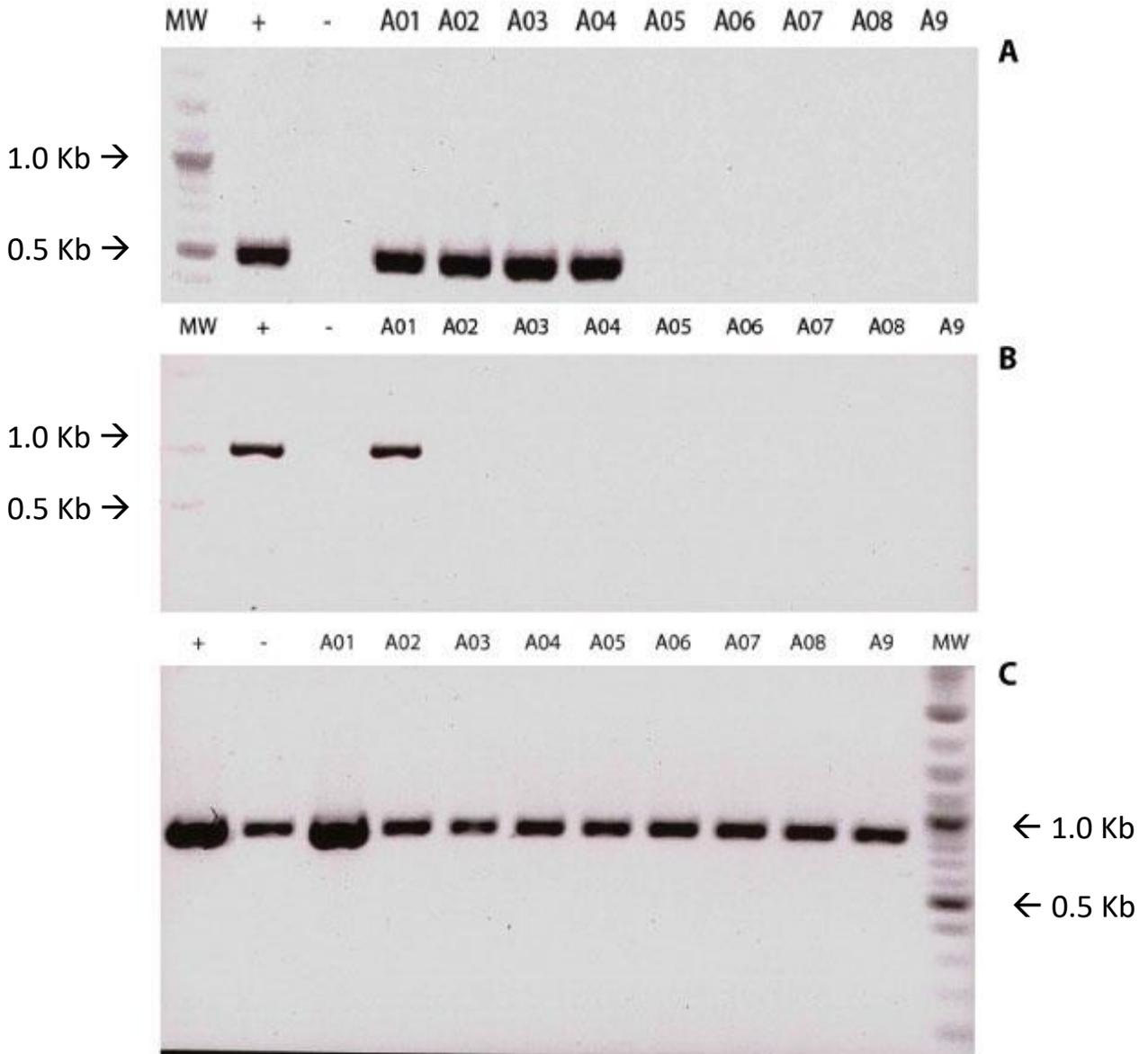


Figure 3. Reverse transcription PCR results for Killer variants A01-A08 and A9 **A.** Primers M1 F1 and M1 R6 were used to amplify part of the M1 Killer ORF. Mutants A01-A04 contain the ORF: mutants A05-A08 and A9 are K^o. **B.** The entire M1 Killer ORF was amplified using primers M1 F5 and M1 R6. Only the Killer ORF in variant A01 was successfully amplified. Variants A02-A04 may contain an upstream mutation that inhibits amplification. **C.** Part of the L-A-1 Helper ORF was amplified using primers LA F2 and LA R2. Variants A01-A08 and A9 all contain a copy of L-A-1 virus.

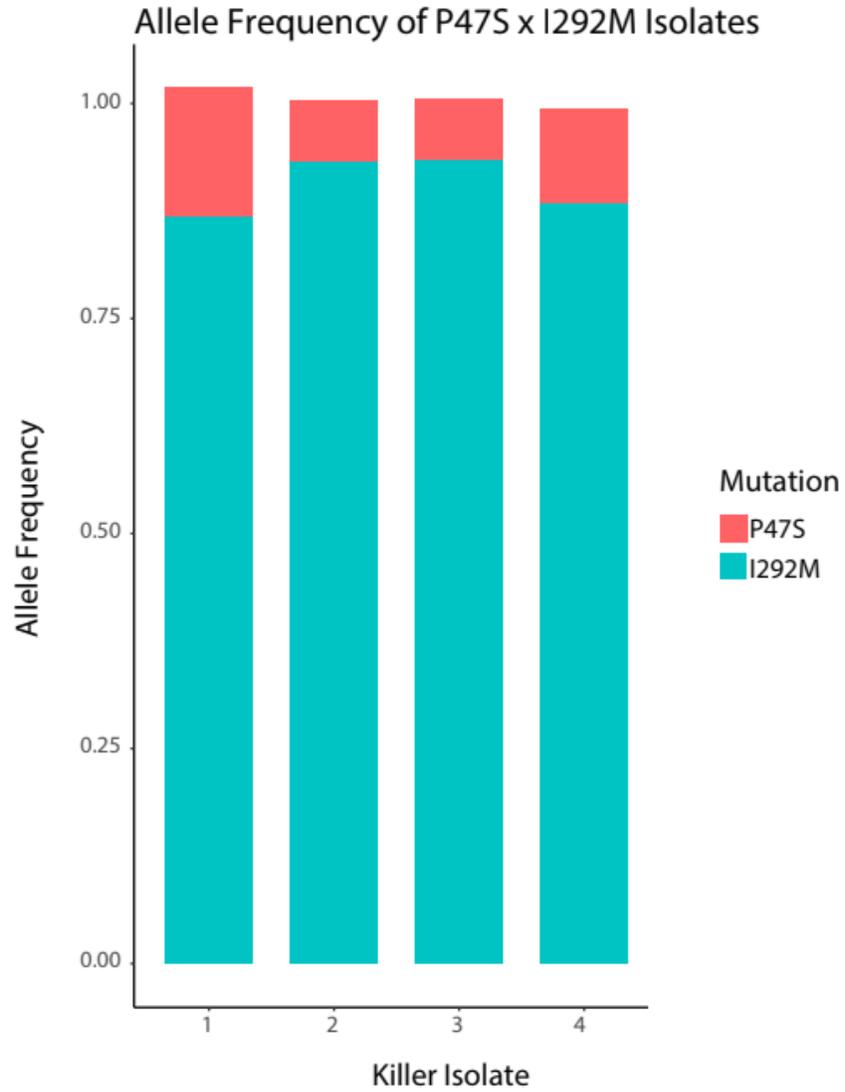


Figure 4. P47S and I292M allele frequencies in four diploid colonies isolated from the P47S x I292M Killer mutant cross. I292M is present at very high frequency amongst all isolates

Table 2. All M1 Killer virus variants isolated in this study

Killer Mutant	Parent Strain	Mating Type	Conditions	Killing phenotype	Resistance Phenotype
A01	yGIL432	<i>MATa</i>	YPD (pH 5.5) @ 23°C	weak killing	resistant
A02	yGIL432	<i>MATa</i>	SD-URA + 5FOA (pH 6.0) @30°C	no killing	sensitive
A03	yGIL432	<i>MATa</i>	SD-URA + 5FOA (pH 6.0) @30°C	no killing	sensitive
A04	yGIL432	<i>MATa</i>	SD-URA + 5FOA (pH 6.0) @30°C	no killing	sensitive
A05	yGIL432	<i>MATa</i>	SD-URA + 5FOA (pH 6.0) @37°C	no killing	sensitive
A06	yGIL432	<i>MATa</i>	SD-URA + 5FOA (pH 6.0) @23°C	no killing	sensitive
A07	yGIL432	<i>MATa</i>	SD-URA + 5FOA (pH 6.0) @30°C	no killing	sensitive
A08	yGIL432	<i>MATa</i>	SD-URA + 5FOA (pH 6.0) @37°C	no killing	sensitive
A1	yGIL432	<i>MATa</i>	BMB YPD (pH 6.0) @23°C	no killing	inconclusive
A2	yGIL432	<i>MATa</i>	BMB YPD (pH 6.5) @ 30°C	weak killing	resistant
A3	yGIL432	<i>MATa</i>	BMB YPD (pH 6.0) @ 23°C	no killing	sensitive
A4	yGIL432	<i>MATa</i>	YPD (pH 5.5) @ 30°C	no killing	sensitive
A5	yGIL432	<i>MATa</i>	MOPS YPD (pH 6.0) @ 23°C	weak killing	resistant
A6	yGIL432	<i>MATa</i>	MOPS YPD (pH 6.5) @ 23°C	no killing	resistant

A7	yGIL432	<i>MATa</i>	MOPS YPD (pH 6.0) @ 23°	no killing	resistant
A8	yGIL432	<i>MATa</i>	YPD (pH 5.5) @ 23°C	no killing	resistant
A9	yGIL432	<i>MATa</i>	MOPS YPD (pH 6.0) @ 23°	no killing	resistant

TABLE 3. All yeast strains used in this study

Yeast Strain	Description	Killer Genotype
yGIL 432	<i>MATa, ade2-1, CAN1, his3-11, leu2-3,112, trp1-1, URA3, bar1Δ::ADE2, hmlαΔ:: LEU2, GPA1::NatMX, ura3Δ::PFUS1-yEVENus</i>	K ⁺
yGIL 1253	<i>MATa, ade2-1, CAN1, his3-11,15 leu2-3,112, trp1-1, URA3, bar1Δ::ADE2, hmlαΔ:: LEU2, GPA1::KanMX, ura3Δ::PFUS1-yEVENus)</i> 1253 variant with KanMX insterted at GPA1 instedad of NatMX	K ^o
yGIL 439	<i>MATa, ade2-1, CAN1, his3-11, leu2-3,112, trp1-1, URA3, bar1Δ::ADE2, hmlαΔ:: LEU2, GPA1::NatMX, ura3Δ::PFUS1-yEVENus</i>	K ^o
yGIL 1097	<i>MATα, ade2-1, CAN1, his3-11,15, leu2-3,112, trp1-1, bar1Δ::ADE2, hmlαΔ:: LEU2, GPA1::NatMX, ura3Δ::PFUS1-yEVENus</i>	K ^o (hypersensitive to Killer toxin)
P47S	Killer variant isolated in yGIL 432	K ⁻
D106G	Killer variant isolated in yGIL 432	K ⁻
I292M	Killer variant isolated in yGIL 432	K ⁻
119FS	Killer variant isolated in yGIL 432	K ⁻
yGIL 1479	<i>MATα, ade2-1, CAN1, his3-11,15 leu2-3,112, trp1-1, bar1Δ::ADE2, hmlαΔ:: LEU2,</i>	K ⁻

	<i>GPA1::NatMX, ura3Δ::PFUS1-yEVENUS) MATα</i> version of D106G	
yGIL1483	<i>MATα, ade2-1, CAN1, his3-11,15 leu2-3,112,</i> <i>trp1-1, bar1Δ::ADE2, hmlαΔ:: LEU2,</i> <i>GPA1::NatMX, ura3Δ::PFUS1-yEVENUS) MATα</i> version of I292M	K ⁻
yGIL 1485	<i>MATα, ade2-1, CAN1, his3-11,15 leu2-3,112,</i> <i>trp1-1, bar1Δ::ADE2, hmlαΔ:: LEU2,</i> <i>GPA1::NatMX, ura3Δ::PFUS1-yEVENUS) MATα</i> version of 119FS	K ⁻
yGIL 1489	<i>MATα, ade2-1, CAN1, his3-11,15 leu2-3,112,</i> <i>trp1-1, bar1Δ::ADE2, hmlαΔ:: LEU2,</i> <i>GPA1::NatMX, ura3Δ::PFUS1-yEVENUS) MATα</i> version of P47S	K ⁻
yGIL 354	<i>MATα, met6Δ::KanMX MATα</i> mating type tester	K ^o
yGIL 353	<i>MATα, met6Δ::KanMX MATα</i> mating type tester	K ^o
