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Experimental tests of host–virus coevolution in natural killer yeast strains

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Abstract

Fungi may carry cytoplasmic viruses that encode anticompetitor toxins. These so-called killer viruses may provide competitive benefits to their host, but also incur metabolic costs associated with viral replication, toxin production and immunity. Mechanisms responsible for the stable maintenance of these endosymbionts are insufficiently understood. Here, we test whether co-adaptation of host and killer virus underlies their stable maintenance in seven natural and one laboratory strain of the genus *Saccharomyces*. We employ cross-transfection of killer viruses, all encoding the K1-type toxin, to test predictions from host–virus co-adaptation. These tests support local adaptation of hosts and/or their killer viruses. First, new host–virus combinations have strongly reduced killing ability against a standard sensitive strain when compared with re-constructed native combinations. Second, viruses are more likely to be lost from new than from original hosts upon repeated bottlenecking or the application of stressful conditions. Third, host fitness is increased after the re-introduction of native viruses, but decreased after the introduction of new viruses. Finally, rather than a trade-off, original combinations show a positive correlation between killing ability and fitness. Together, these results suggest that natural yeast killer strains and their viruses have co-adapted, allowing the transition from a parasitic to a mutualistic symbiosis.

Introduction

Host-symbiont relations are many. Examples include legume roots and their nitrogen-fixing rhizobia (Gage, 2004), marine sponges and their bacterial communities (Webster & Taylor, 2012), insects and their Wolbachia endosymbionts (Serbus *et al.*, 2008), bacteria and their plasmids (Bouma & Lenski, 1988), and animals and their gut microbiomes (Hongoh, 2010, 2011; Marchesi, 2010). The association between host and symbiont may vary from facultative to obligate, depending on the strength of the dependence of partners on each other. In a mutualistic symbiosis, this dependence is high and mutual (Nyholm & Graf, 2012), but has presumably evolved from initially more loose or parasitic interactions (Aanen & Bisseling, 2014). However, it is often unknown whether and how coevolution of both partners has shaped the observed symbiosis.

Yeast killer strains provide an interesting example of a mutualistic symbiosis. So-called killer phenotype is based on the production and secretion of low-molecular mass proteins and glycoprotein toxins (Makower & Bevan, 1963), which kill sensitive strains of the same and closely related species or genera (Schmitt & Breinig, 2006). Killer systems are noninfectious and apparently symptomless in their typical hosts (Ghabrial, 1998). For example, yeast cells of the genus *Saccharomyces* host cytoplasmic M viruses, which encode anticompetitor toxins and corresponding immunity components, and LA helper viruses, which are responsible for encoding capsid proteins and the viral RNA-dependent RNA polymerase (Schmitt & Breinig, 2002, 2006; McBride *et al.*, 2013). Yeast killer strains have been found in nearly every environment tested: fruits,

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mushrooms, spontaneous fermentation, soil, decaying plant material, and industrial and laboratory collections (Schmitt & Breinig, 2002). In a screen of more than one hundred isolates of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* obtained from laboratory collections, nature, vineyards, clinics and industry, we found that about 10% carried killer viruses, whereas approximately 25% of the strains were resistant to viral toxins, confirming that killer viruses are a significant factor in the evolution of *Saccharomyces* yeasts (Pieczynska *et al.*, 2013).

Little is known about the evolutionary forces responsible for the maintenance of yeast killer strains. Yeasts hosting killer viruses may benefit from toxin production when competing with other yeasts that do not carry killer viruses by securing primary resources and liberating additional resources from killed competitors (Wloch-Salamon *et al.*, 2008). Conversely, killer viruses depend entirely on their host, because transfer of the yeast killer viruses is strictly vertical from mother to daughter cell, with the exception of sexual mating. Therefore, the killer viruses are inherited either after cell division, during sporogenesis or through mating with a donor cell. Furthermore, they cannot escape their host and infect new ones (Wickner, 1996), except during rare outcrossing events (Zeyl & Otto, 2007). Virus carriage initially incurs a fitness cost associated with the metabolic costs of viral replication, toxin production and immunity (Wloch-Salamon *et al.*, 2008). Therefore, in the absence of toxic killing, selection may cause the loss of the killer virus. On the other hand, as virus fitness depends strongly on host fitness, fitness costs of carrying the virus are expected to diminish over time (McBride *et al.*, 2013).

Either adaptation of the host to its killer virus, adaptation of the killer virus to its host or both can stabilize the association; only in the latter case, host and virus are said to have co-evolved (Janzen, 1980). One possibility is that compensatory mutations removing the cost of viral carriage have benefits that are conditional on the presence of the virus. This was seen in a recent laboratory evolution study with killer yeast (Pieczynska *et al.*, 2016), where we observed the rapid evolution of a partial dependence of host fitness on the presence of the virus, despite the initial cost of viral carriage. These results showed the potential for co-adaptation in the yeast killer system under experimental conditions in the laboratory. However, little is known about the eco-evolutionary forces affecting this symbiosis in nature.

Here, we look for signatures of adaptation between yeast hosts and their toxin-encoding viruses in natural yeast killer strains. We transferred viruses among eight killer strains, including seven wild (Liti *et al.*, 2009; Schacherer *et al.*, 2009) and one laboratory strain (Wloch-Salamon *et al.*, 2008), and tested how in the new host–virus combinations the killing phenotype, competitive fitness and stability of the host–virus association are affected. Our results show clear signs of local adaptation of hosts and/or their viruses in all tests performed, as the new combinations had lower killing abilities, higher viral loss rates and lower competitive ability. Remarkably, rather than a trade-off, we find a positive relationship between competitive fitness and killing ability among original killer strains. Finally, we find that strains transfected with viruses from hosts belonging to the same species show higher killing ability than those transfected with viruses from hosts of a different species.

Materials and methods

Strains

Table 1 lists all strains used. These include a previously constructed K1 killer and isogenic (except for selectable

Table 1 Cross-transfected strains used in the experiment.

Donor\Acceptor	Q62.5	Q74.4	T21.4	Y8.5	YJM454	CLIB294	SK1	Lab.K1	Sensitive
Q62.5 *Saccharomyces paradoxus*	–	–	+	+	+	–	+	+	+
Q74.4 *S. paradoxus*	–	–	+	+	+	–	+	+	+
T21.4 *S. paradoxus*	–	–	+	+	+	–	+	+	+
Y8.5 *S. paradoxus*	–	–	+	+	+	–	+	+	+
YJM454 *Saccharomyces cerevisiae*	–	–	+	+	+	–	+	+	+
CLIB294 *S. cerevisiae*	–	–	+	+	+	–	+	+	+
SK1 *S. cerevisiae*	–	–	–	–	–	–	–	–	–

Plus scores indicate successful transfections, and minus scores indicate failed transfections.

markers) toxin-sensitive strain of *Saccharomyces cerevisiae* (Wloch-Salamon *et al.*, 2008) and seven wild killer strains of *S. cerevisiae* and *S. paradoxus* derived from two yeast strain collections (Liti *et al.*, 2009; Schacherer *et al.*, 2009). The seven wild killer strains were found in natural habitats, distilleries and clinics and all harboured viruses encoding the K1-type toxin (Pieczynska *et al.*, 2013). The laboratory K1 killer strain and sensitive strain serve as a reference for the killing ability of the K strains and the effect of curing strains from their viruses, respectively. A toxin-resistant strain was used as reference in competition assays to measure fitness in the absence of killing benefits (Pagé *et al.*, 2003; Wloch-Salamon *et al.*, 2008).

Media

Liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used to grow strains prior to all experiments listed below. Low-pH liquid YPD (YPD adjusted with a phosphate-citrate buffer to pH = 4.6) was used for the 'equilibration experiment'. Low-pH YPD solidified with 2% agar was used for the competition experiments and for assays using single-cell transfers. To estimate competitor numbers in the fitness assays, SC (synthetic complete) medium with 1% 5-FOA was used to score colonies with uracil auxotrophy (inability to synthesize uracil and therefore grow in media without uracil) and SC medium without uracil to score for colonies with uracil prototrophy (ability to produce uracil). SC without uracil was also used to select for transformants in the transfection experiments. Assays of killing ability, toxin sensitivity so-called halo assays, were performed with low-pH YPD supplied with 0.003% MB (methylene blue) and solidified with 2% agar.

Curing killer strains from their viruses

Prior to starting cross-transfection manipulations, all strains were subjected to the standard protocol of virus curing via propagation on YPD agar plates for 3 days at an elevated temperature (38 °C for most of the strains and 40 °C for strain YJM454, which was unable to be cured at 38 °C) (Wickner, 1974; Pieczynska *et al.*, 2013). Next single colonies (10 for each strain) were screened for the presence of a killer phenotype using a sensitive reference strain, and toxin sensitivity using a laboratory K1 killer strain, based on the standard halo method (Kishida *et al.*, 1996).

Isolation of killer viruses

Donor strains were grown in 500 mL of liquid YPD medium for 3–4 days at 30 °C. Cells were collected by low-speed centrifugation (3000 *g*), washed with the SEKS buffer (1 M sorbitol, 0.1 M EDTA, 0.1 M Na_2SO_4, 0.8 M KCl, pH = 7.5) and suspended in 10 mL of the PKE buffer (30 mM Na_2HPO_4 150 mM KCL, 10 mM EDTA, pH = 7.6). Cells were treated with 0.1–1% non-ionic detergent (Np40) and incubated for 1 h at 30 °C. Disrupted cells were centrifuged at 4000 *g* for 30 min at 4 °C to separate supernatant from cell debris. The supernatant was fractionated in a 30% sucrose cushion by centrifugation for 2.5 h at 32 000 *g* at 4 °C. The resulting pellet, containing killer viruses, was suspended in PKE buffer. Suspensions were immediately used for cross-infections or stored at −80 °C.

Cross-transfection of killer viruses

The *pAG60* plasmid with the selectable *URA3* gene (Goldstein *et al.*, 1999) was used to check for successful cross-transfections. Laboratory killer and sensitive strains were uracil auxotrophs, whereas all wild killers were originally prototrophs, and therefore, their chromosomal copy of *URA3* was replaced with the cassette derived from the toxin-sensitive strain using the lithium acetate procedure (Gietz *et al.*, 1995). To begin cross-transfections, cells were collected from exponentially growing cultures by low-speed centrifugation (3000 *g*) and washed four times with water. Cells were then suspended in 1 M LiAc and immediately collected by centrifugation at 13 000 *g* for 30 s. Cells were then suspended in the transformation mix containing 240 μL PEG 3500 50% w/v, 36 μL 1 M LiAc, 50 μL ssDNA, 5 μL of the *pAG60* plasmid and 100 μL of supernatant containing viruses. This mixture was incubated for 10 min on ice, followed by 50 min at 30 °C, and 10 min at 37 °C. The cells were collected by centrifugation for 30 s at 8000 *g* and suspended in YPD and immediately spread on SC-uracil plates. After 3 days of incubation at 30 °C, colonies were picked, followed by the assessment of killing ability and toxin sensitivity. Single clones showing the killer phenotype and toxin resistance, thus carrying killer viruses, were stored at −80 °C.

Each transfected strain (with either its own or a foreign virus) was cultured for eight serial transfers (~50 generations) under standard growth conditions that were optimal for the production and activity of the K1 toxin (YPD with pH 4.6, 25 °C), to allow the new combinations to physiologically equilibrate. If not indicated otherwise, all reported assays were carried out with frozen samples from after this equilibration phase.

Assay of killing ability

Low-pH MB-YPD agar plates were inoculated with 200 μL of a 100-fold dilution of YPD stationary-phase cultures of sensitive cells (~4×10^5 cells per plate). After the plates dried up, three replicates of 5 μL aliquots of undiluted (~2×10^8 cells mL^{-1}) overnight killer cultures (grown from freezer stocks derived from single clones) were overlaid as small central patches.

The size of the zone of growth inhibition (or halo) produced around the K patch was measured manually after 72 h of incubation at 25 °C, and killing ability was expressed as the total surface area of the halo (i.e. surface area of the zone of no growth surrounding the killer patch) divided by the surface area of the killer patch.

Assay of toxin sensitivity

Low-pH YPD agar plates supplied with 0.003% MB were inoculated by depositing 50 μL aliquots of a 100-fold dilution of the YPD stationary-phase culture of tested killer (cured or after transfection with viruses). After the patches dried up, three replicated of 5 μL aliquots of undiluted (~2×10^8 cells mL^{-1}) overnight laboratory K1 cultures were put as small patches onto the tested killer patches. The presence of the halo formed around the K patch was scored manually after 72 h of incubation at 25 °C.

Assay of competitive ability

Relative fitness was measured by pairwise competitions of each strain against a standard toxin resistance reference strain with a different antibiotic-resistant marker (Pagé *et al.*, 2003; Wloch-Salamon *et al.*, 2008). Strains were grown separately by transferring 1% of a stationary-phase culture (~2×10^6 cells) to YPD agar plates (pH = 4.6) and incubating for 24 h at 25 °C, in order to adjust strains to conditions of the competition environment. Cells were then washed off the plates with 10 mL of water, mixed in equal proportion, and then, an aliquot of 10 μL (~2×10^6 cells) was spread on fresh low-pH YPD agar plates and incubated for 48 h at 25 °C. The frequencies of both competitors were estimated by plating dilutions of washed-off cells on selective agar media before and after competition and counting colonies of both types after 48 h of incubation. Relative fitness of each strain was calculated as the ratio of its Malthusian parameter to that of a reference resistant strain (Lenski *et al.*, 1991). Three independent replicate assays of each competition experiment were performed per strain.

Assessment of the stability of host–virus associations

Both original and newly constructed killer strains were faced with three conditions that are known to increase the rate of virus loss. One condition was an elevated temperature (all strains tested at 38, 40 and 42 °C) (Wickner, 1974). Strains were grown on YPD agar plates for 3 days, after which single colonies (ten for each strain) were screened for the presence of the killer phenotype with the standard halo method (Kishida *et al.*, 1996). Second, three concentrations of cycloheximide (0.3, 0.5, 1 μg mL^{-1}) were applied (Fink & Styles, 1972). Again, strains were grown on YPD agar plates supplemented with cycloheximide for three consecutive days followed by the halo test applied to randomly selected single clones. Finally, killer strains were passed serially through 10 single-cell transfers to minimize effects of selection between host cells carrying varying titres of viruses. This was carried out on YPD agar with three replicate lines per strain by streaking single colonies every 72 h on fresh medium (allowing 20–25 generations during colony growth between transfers). Viral loss was determined for all strains and conditions using the halo test vs. a standard sensitive strain (killing ability test) and laboratory killer strains (sensitivity assay), where a complete absence of a halo in the first test, followed by the presence of a halo around the cured killer in the sensitivity test, was scored as viral loss.

Results

We performed transfection of toxin-encoding viruses of natural killer strains to test for adaptation between host and virus. Seven natural virus-carrying strains from various sources were used as donors; these seven strains, together with two laboratory strains (one with, the other without the virus), were also used as recipients, after removal of their viruses (Table 1). The seven natural strains came from collections of *S. cerevisiae* and *S. paradoxus* strains with fully sequenced genomes (Liti *et al.*, 2009; Schacherer *et al.*, 2009), from which we recently identified these seven strains, all carrying killer viruses of the common K1 type (Pieczynska *et al.*, 2013). The viruses and virus-cured host strains were used in an attempt to construct all 63 (i.e. seven donors and nine recipients) possible donor–recipient combinations. Only 36 transfections were successful due to problems either with viral isolation or transfection (Table 1). Specifically, we were unable to isolate viruses from one of the wild strain (SK1). Three strains (Q62.5, Q74.4 and CLIB294) could not be transfected with any of the viruses, including their own, despite positive control transformations with a plasmid. Halo assays, where transfected strains were confronted with the laboratory K1 killer strain, indicated that they were still fully sensitive to the killer toxin, confirming the absence of killer virus. With this collection of native and newly constructed killer strains, we performed three tests.

Killing ability

We first analysed the killing ability of original and transfected strains against a reference toxin-sensitive strain (see Fig. S1). To test the quality of our transfection method, we compared the killing ability of the three strains for which reconstruction was successful

(T21.4, Y8.5 and YJM454). Surprisingly, immediately after curing and re-transfection with their own virus, these three strains showed lower killing ability compared to the original strains (Fig. 1a). However, culturing the transfected strains for about 50 generations under optimal conditions for the production and activity of the K1 toxin largely recovers their killing ability (Fig. 1a; two-tailed $P > 0.10$ for all three strains using two-sample *t*-tests). This confirms that our transfection method is basically sound if we take this equilibration period into account.

We then asked whether killer viruses show lower killing ability in other hosts than their own, which would suggest local adaptation of host or virus or both. Similar as for the three original combinations in Fig. 1a, killing ability is lower after transfection of the virus in new hosts (Fig. 1b). However, killing ability of these new host–virus combinations does not increase after the 50-generation equilibration period: one-sample *t*-tests comparing mean killing ability for each virus in its original host with that in five or six new hosts confirm that performance remains significantly lower for new host–virus combinations (two-tailed $P < 0.005$ for all six viruses). The contrast in performance between reconstructed original and new host–virus combinations therefore suggests local adaptation of host and/or virus. Given that the 50-generation equilibration period after transfection is essential for obtaining original performance, all reported further assays were performed after equilibration.

As the killer strains came from two species of *Saccharomyces* (*S. paradoxus* and *S. cerevisiae*), we tested whether the reduction in killing ability was smaller for new combinations made within, relative to between, these two species. Figure 2 shows that indeed the 13 within-species transfections (six for *S. paradoxus* and seven for *S. cerevisiae*) yielded higher estimates of killing ability than the 20 transfections between species (four for *S. paradoxus* and 16 for *S. cerevisiae* as recipient

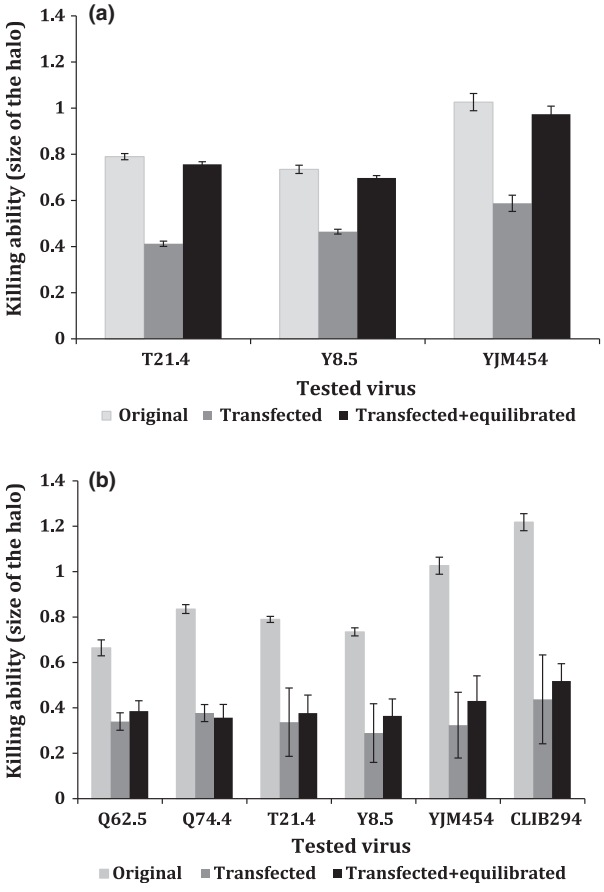

Fig. 1 Killing ability of native and newly constructed killer strains measured against a reference toxin-sensitive strain. (a) Killing ability of the three strains where, after virus curing, the native virus was successfully reintroduced; light grey bars indicate original performance, dark grey bars indicate performance immediately after transfection, and black bars indicate performance of the reconstructed host–virus combinations after 50 generations of equilibration. Error bars reflect standard errors of the mean based on three independent measurements. (b) Killing ability of the six strains that were successfully transfected with virus from other hosts; light grey bars are for original performance, dark grey bars indicate performance immediately after transfection, and black bars are for the new host–virus combinations after 50 generations of equilibration. Error bars are standard errors of the mean based on three independent measurements per strain (original combinations) or five or six mean estimates (newly constructed combinations).

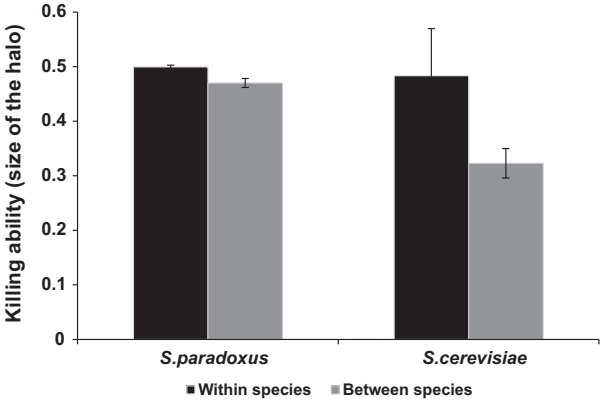

Fig. 2 Killing ability of strains created by transfection with viruses between host strains of the same (black) or different (dark grey) yeast species, separately for *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* hosts. Killing ability was measured of transfected (and equilibrated) strains against a standard toxin-sensitive strain with three-fold replication for 13 within-species (six for *S. paradoxus* and seven for *S. cerevisiae*) and 20 between-species transfections (four for *S. paradoxus* and 16 for *S. cerevisiae*). Error bars represent standard errors of the mean of within and between-species combinations for the two species.

host). To test whether host–virus combinations from the same species showed higher levels of co-adaptation than combinations from different species, we used the 33 allopatric host–virus assemblies and tested for the effects of the two yeast species as a random factor, and between vs. within-species as fixed factor, which showed a significant effect of the latter term ($F = 6.42$, d.f. = 1.29, $P = 0.017$).

Host fitness

As a next test of host–virus co-adaptation, we considered the effect of viral transfection on the competitive ability of the hosts. We performed competition experiments between original, cured and re-constructed killer strains against a toxin-resistant reference strain on standard YPD agar medium in the absence of sensitive cells (i.e. without possible benefits of toxic killing). The assays were carried out using agar instead of liquid cultures to make results comparable with those of a previous study (Wloch-Salamon *et al.*, 2008). Figure 3a first shows the relative competitive ability of laboratory killer and sensitive reference strain before and after curing. The curing procedure itself did not affect competitive ability (two-sample *t*-test of fitness before and after curing of the sensitive strain: $t = 0.019$, d.f. = 4, two-tailed $P = 0.986$). However, consistent with previous results (Wloch-Salamon *et al.*, 2008), curing the constructed laboratory killer strain, which had limited opportunity for host–virus coadaptation, caused a fitness increase of ~7% ($t = 7.10$, d.f. = 4, $P = 0.0021$), which was indistinguishable from that of the sensitive strain ($t = 1.24$, d.f. = 4, two-tailed $P = 0.283$).

To measure the effect of curing the natural killer strains from their viruses and introducing new viruses on competitive fitness, we measured fitness of the six wild killer strains after curing and after transfection for four of the strains for which transfection with virus from strains T21.4 and Q74.4 was successful (Fig. 3b). Rather than causing a fitness increase, as for the constructed laboratory killer strain, curing the natural killers from their virus caused on average a ~11% fitness decrease (paired *t*-test: $t = 9.53$, d.f. = 5, two-tailed $P < 0.001$). Moreover, the introduction of viruses from strains T21.4 and Q74.4 to new hosts caused a further fitness decline of ~5% relative to cured status (paired *t*-test: $t = 9.35$, d.f. = 6, two-tailed $P < 0.001$), whereas reconstruction of strain T21.4 brought fitness back to the original level ($t = 0.017$, d.f. = 4, $P = 987$), as was observed for its killing ability (Fig. 1a). These results are consistent with the reported declines in killing ability for novel host–virus combinations (Fig. 1b) and indicate adaptation of hosts to their own killer virus, or vice versa, or both.

Because the introduction of a new virus initially incurs a fitness cost (Fig. 3a), we then asked whether

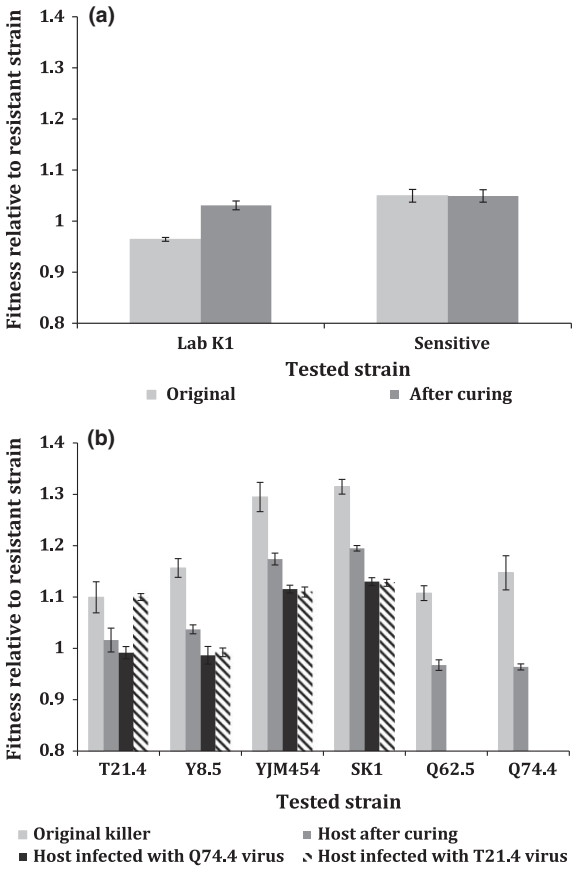

Fig. 3 Fitness of host strains carrying original viruses measured in competition against a reference toxin-resistant strain on standard YPD agar medium (light grey). (a) Fitness of laboratory killer and sensitive strain, before (light grey) and after curing (dark grey). (b) Fitness of the six hosts that were successfully transfected, before (light grey) and after curing from their original viruses (dark grey), as well as after introduction of two new viruses: Q74.4 (black), T21.4 (white with black stripes). Competition experiments with newly constructed combinations were performed after 50 generations of equilibration. Error bars represent standard errors of the mean based on three independent assays.

fitness and killing ability still show a trade-off in the native host–virus combinations. We examined this for the seven natural killer strains and the constructed K1 killer strain. Figure 4 shows that, rather than a negative correlation, killing ability and fitness correlate positively (Pearson's $r = 0.794$, $n = 8$, $P = 0.018$). Taking into account that the constructed K1 has not shared any evolutionary history with its virus, we also tested the correlation for wild killer strains only (Pearson's $r = 0.820$, $n = 7$, $P = 0.024$). Apparently, whatever fitness cost the viruses incurred initially, these were removed by subsequent adaptation of either host or virus, or both.

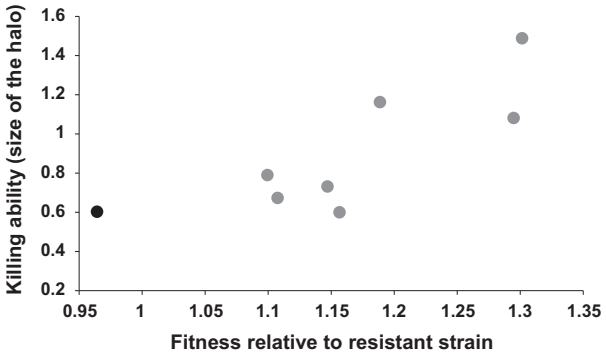

Fig. 4 Relationship between killing ability and competitive fitness for the eight original killer strains. Fitness was measured in direct competition experiments against a toxin-resistant reference strain under standard laboratory conditions. The seven natural killer strains are shown in light grey, the laboratory killer strain (Lab.K1) in black.

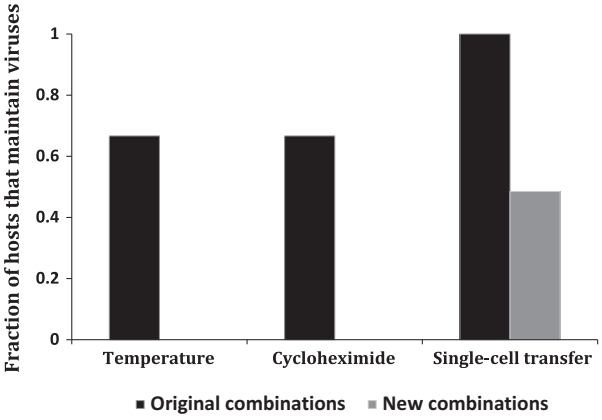

Fig. 5 Stability of host–virus associations in reconstructed original and new combinations after equilibration using three different stress conditions (growth at high temperature or in the presence of cycloheximide and 10 single-cell transfers). Shown is the fraction of tested samples showing viral maintenance for the three original (black) and 33 new combinations (dark grey; for temperature and cycloheximide stress no samples showed viral maintenance). Viral maintenance was scored by the presence of a halo using halo tests with the reference toxin-sensitive strain.

Stability of host–virus associations

Finally, we hypothesized that adaptation of hosts and viruses may have increased the stability of their association, either due to high virus titres or, indirectly, due to their greater toxicity and hence stronger selection against cells losing their viruses. We tested this by comparing rates of viral loss for three re-constructed native and 33 new host–virus combinations under three conditions known to enhance virus loss: elevated temperature, growth in the presence of cycloheximide and repeated single-cell bottlenecking. Virus presence was tested using the halo test. We noted that at 38 °C and a cycloheximide concentration of 0.3 μg mL^{-1} differences in viral loss were most pronounced, and used these to score viral loss. Figure 5 shows that the three native host–virus combinations were significantly more stable under elevated temperature, cycloheximide application and single-cell transfers than the 33 new combinations ($P = 0.021$, $P = 0.006$, and $P = 0.002$, respectively, using Fisher's exact probability test on the frequency of viral loss among the old vs. new combinations). The clearest difference in stability was observed in the strains that underwent single-cell transfers, where viruses were lost in more than half of newly created combinations, but in none of reconstructed original combinations.

Discussion

We performed cross-transfection experiments with seven wild and one laboratory yeast killer strains belonging to two species (*S. cerevisiae* and *S. paradoxus*) to test for signs of adaptation between host and virus. All eight strains harboured the M virus-like particle encoding the K1 toxin together with LA helper virus in their cytoplasm and were able to kill cells of a standard sensitive reference strain under certain conditions. By exchanging killer viruses among these eight strains, we were able to show that toxicity is higher for original than for newly constructed host–virus combinations, as well as for newly created combinations within vs. between the two yeast species. In addition, competitive ability in the absence of toxic killing was also lower for new than original host–virus combinations. Remarkably, we found that loss of the virus had a positive effect on the competitive ability of the constructed laboratory killer strain, indicating initial fitness costs of viral carriage, whereas it had a negative effect on fitness in the natural killer strains. Finally, the rate of viral loss during conditions of extreme genetic drift or stress was higher for newly created than for original host–virus combinations. Together, these results suggest that host and virus have co-adapted in natural killer strains.

A complicating factor for comparing performance of original and new host–virus combinations was that new combinations showed suboptimal performance immediately after transfection. However, killing ability increased after 50 generations of growth under benign conditions, which allowed killing abilities (Fig. 1 and Fig. S1) and competitive fitness (Fig. 3b) to reach similar levels as those of the original strains. We do not know the reason, but speculate that the lower performance immediately after transfection may have been due to the effective transformation of few viral particles (only a fraction of the viral supernatant was used for

each transfection), and improved performance after 50 generations of growth is due to the equilibration of virus titres and/or gene expression. We cannot rule out that compensatory mutations contributed to the observed improved performance, but changes in virus titres or epigenetic modifications seem more likely during only 50 generations of growth. We found no signs that transfections with native viruses were more often successful than transfections with foreign viruses (see Table 1), because technical problems with virus isolation and transfection overruled these more subtle signals of local adaptation. However, it is conceivable that selection has differentially affected virus titres of original and new host–virus combinations, given that original combinations have higher fitness, whereas new combinations have slightly lower fitness relative to cured strains (Fig. 3b).

The negative fitness effect of removing killer viruses from the natural strains, despite initial fitness costs of virus carriage revealed in the constructed killer strain (Fig. 3), exemplifies that host and virus have become mutualistic symbionts. McBride *et al.* (2013) showed that the loss of LA helper and M viruses led to genome-wide alterations in gene expression of the yeast host, indicating that coevolution between virus and yeast has led to adjustments in host metabolism. Adaptation in one or both symbionts leading to the mutual (partial) dependence of symbionts has been observed in many other systems. For example, in laboratory evolution studies with bacteria and plasmids, bacteria were shown to evolve dependence on their plasmids, when these carry genes encoding toxins with a longer half-life than that of the antidote they also encode (Van Melderen & De Bast, 2009), or after compensatory mutations for the metabolic cost of plasmid carriage occur in the bacterial genome that are deleterious in the absence of the plasmid (Bouma & Lenski, 1988). Analogously, compensatory evolution has been frequently observed within the same genome in antibiotic-resistant bacteria (Andersson & Hughes, 2010) and toxin-resistant fungi (Schoustra *et al.*, 2007), where initial fitness costs of toxin resistance are removed during laboratory evolution, sometimes also leading to decreased fitness after removal of the resistance mutation (Schoustra *et al.*, 2007). Other support for host-endosymbiont adaptation was found for Wolbachia bacteria and their insect hosts, where hosts were shown to become infertile after removal of the endosymbiont (Pannebakker *et al.*, 2007).

Interactions between coevolving symbionts are often antagonistic when the fitness of one partner only partially depends on the fitness of the other partner (Van Valen, 1973; Stenseth & Smith, 1984). When stronger dependence evolves, such as for endosymbionts being unable to spread to other hosts, natural selection acting at the level of the symbiont combination is expected to limit further antagonism (Szathmáry & Smith, 1995). This transition from antagonism to mutualism also seems to have happened in the yeast killer system. The killer virus has become almost entirely dependent on its host, as horizontal transmission to new hosts has become very infrequent: no extracellular route of infection is known (Wickner, 1996) and outcrossing happens at a very low frequency (Zeyl & Otto, 2007). The dependence of the host on its killer virus is not vital, but still significant: the killer virus enlarges the habitat of yeast by allowing for killing of resource competitors and removal of the virus incurs a fitness cost even in the absence of competitive benefits from toxic killing.

It seems likely that adaptive changes occurred in both host and virus during their shared evolutionary history, but they remain hypothetical without temporal information (Janzen, 1980). Support that both symbionts co-evolved comes from the fact that both host and virus identity affected killing ability, whereas native combinations showed the highest performance, and fitness even decreased after removal of the native viruses. However, we cannot rule out that this variation existed before these symbioses were established or that the genetic changes occurred in only one symbiont or in response to the abiotic environment rather than in response to the other partner. In a recent laboratory evolution experiment, we demonstrated the reciprocal nature of changes in both host and killer virus relative to their ancestral states (Pieczynska *et al.*, 2016). It is therefore likely that the signs of adaptation between host and virus observed in natural killer strains in our present study involved similar reciprocal changes, but only over longer time periods.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Figure S1 Killing abilities of all successful new and original host-virus combinations.

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