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Rapid multiple-level coevolution in experimental populations of yeast killer and nonkiller strains

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Coevolution between different biological entities is considered an important evolutionary mechanism at all levels of biological organization. Here, we provide evidence for coevolution of a yeast killer strain (K) carrying cytoplasmic dsRNA viruses coding for anti-competitor toxins and an isogenic toxin-sensitive strain (S) during 500 generations of laboratory propagation. Signatures of coevolution developed at two levels. One of them was coadaptation of K and S. Killing ability of K first increased quickly and was followed by the rapid invasion of toxin-resistant mutants derived from S, after which killing ability declined. High killing ability was shown to be advantageous when sensitive cells were present but costly when they were absent. Toxin resistance evolved via a two-step process, presumably involving the fitness-enhancing loss of one chromosome followed by selection of a recessive resistant mutation on the haploid chromosome. The other level of coevolution occurred between cell and killer virus. By swapping the killer viruses between ancestral and evolved strains, we could demonstrate that changes observed in both host and virus were beneficial only when combined, suggesting that they involved reciprocal changes. Together, our results show that the yeast killer system shows a remarkable potential for rapid multiple-level coevolution.

KEY WORDS: **Coevolution, experimental evolution, dsRNA virus, killer yeast.**

The term coevolution is used to describe reciprocal adaptation between species or other biological entities. Coevolution may vary in the complexity, involving either two or more species affecting each other's evolution (Thompson 1994). Coevolutionary processes can occur at different levels of biological organization, including host plants and their pollinating butterflies (Merrill et al. 2013), ants (Fischer et al. 2002), and wasps (Cook and Rasplus 2003), animals and their gut commensals (Hongoh 2010; Marchesi 2010), hosts and their parasites (Decaestecker et al. 2007; Schulte et al. 2010; Koskella et al. 2011), eukaryotic cells and their mitochondria (Zeyl et al. 2005), as well as bacteria and their bacteriophages or plasmids (Bouma and Lenski 1988; Buckling and Rainey 2002; Forde et al. 2008; Hall et al. 2011; Meyer et al. 2012). It can lead to different evolutionary outcomes where either only one (parasitism) or both partners enjoy benefits (mutualism). The particular outcome of coevolution depends on the relative evolvability of each partner, as well as on the dependence on each other. For instance, the short-generation time and large population size of viruses give them an evolutionary edge over most of their hosts (Buckling et al. 2009). Similarly, the ability of parasites to escape from their present hosts and infect new hosts is an important determinant of their virulence since it affects the relation between virulence and fitness (Aanen and Bisseling 2014).

Viruses, being the most ubiquitous biological entities on Earth and found in almost every habitat, are often obligate parasites. They depend on their hosts whose cellular environment is essential for their survival and replication. Viruses may coevolve with their hosts in many ways, one possible result being a stable endosymbiotic relationship (Ghabrial 1998; Pearson et al. 2009).

EVOLUTION
INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

However, viruses can often escape their present host and spread to new ones, as it happens with bacteria and their horizontally transmitted phages (Buckling and Rainey 2002; Pal et al. 2007; Gandon et al. 2008; Marston et al. 2012). Such interactions may accelerate genome evolution, especially in the genes that encode virulence and host-protection factors (Barrick and Lenski 2013). Some studies on bacteria-phage interactions have shown that since phage tend to evolve faster than bacteria, increased mutation rates enabling faster adaptive responses in the latter may be selected (Pal et al. 2007; Paterson et al. 2010; Brockhurst and Koskella 2013).

An example of a mutualistic relationship between a microbe and a virus is that of yeast and its virus in the so-called *Saccharomyces* killer system (Schmitt and Breinig 2002). Killer strains of the genus *Saccharomyces* carry two separately encapsulated double-strand RNA killer viruses. One is responsible for the production of toxin and antidote, the second encodes capsid proteins and RNA-dependant RNA polymerase (Magliani et al. 1997; Marquina et al. 2002). The anti-competitor toxin is effective against strains that lack the virus elements. Competition via toxins occurs not only in yeast, where it is observed in a wide range of natural habitats (Schmitt and Breinig 2002; Gulbiniene et al. 2004), but is also common in plants (Callaway and Aschehoug 2000), marine invertebrates (Jackson and Buss 1975), bacteria (Adams et al. 1979), and other microbial populations. Interference competition via toxin production is thought to play an important role in the maintenance of microbial diversity (Adams et al. 1979; Czárán et al. 2002; Kerr et al. 2002; Pintar and Starmer 2003). Strains that do not produce toxins and are sensitive to them loose in competition against killers. However, since viral replication and toxin production involve metabolic costs, nonproducers are more effective in competing for resources in the absence of killers (Pintar and Starmer 2003).

The outcome of competition between toxin-producers and nonproducers depends on resource availability and the frequency of their encounters, which in turn depends on the spatial structure of environment, since yeasts are not motile. When dispersal is low, toxin producers benefit more than nonproducers, since they are close to the liberated resources (both primary limiting resources and those released from killed individuals) (Chao and Levin 1981; Amarasekare 2002; Czárán et al. 2002; Kerr et al. 2002; Wloch-Salamon et al. 2008). At higher dispersal rates, the benefits of killing also fall back to individuals not producing toxin and the net benefit of toxin production depends on the frequency of producers: when killer frequencies are too low, toxin concentrations are insufficient to kill sensitive cells (Chao and Levin 1981; Greig and Travisano 2008).

Coevolution may affect yeast killer strains at two distinct levels: driven by interactions between a toxin-producer strain and a nonproducer strain, and by interactions between the yeast host and its killer viruses. As explained above, interactions between killer and sensitive strain involve both resource and interference competition: both strains compete for limiting resources, but only the killer strain is able to interfere directly with nonproducers via toxin killing. Costs associated with toxin production may decline during the coevolution between the virus and its host via mutations that compensate for these costs, analogous to compensatory evolution removing the fitness costs of antibiotic resistance (Andersson and Hughes 2010) or bacterial plasmid carriage (Bouma and Lenski 1988). A former study on killer yeast illustrates that loss of viruses by yeast hosts results in changes in the yeast genome indicating some integration of virus and host metabolism due to coadaptation between them (McBride et al. 2013). We recently found that swapping killer viruses among natural isolates of killer strains demonstrates that, after sufficient evolutionary time, the fitness costs of virus carriage become compensated and may even lead to fitness reduction upon removal of the virus (M. D. Pieczynska, R. Korona, J. A. G. M. de Visser, unpubl. data). However, factors governing the coevolutionary dynamics in the yeast killer system remain poorly known.

Here, we use experimental evolution to study the dynamics of coevolution in the yeast killer system. We are particularly interested in changes in interference and resource competitive ability when killer and sensitive strains interact frequently. We allow populations of constructed *Saccharomyces* killer (K) and isogenic nonkiller (S) strains to evolve for 500 generations in a structured environment in which the opportunity for coevolution can be manipulated. Specifically, we allow either both strains or only one strain to evolve in mixed populations (Rice 1996) and use populations of pure K and S strains as controls. By weekly resetting the ratio of K and S strain in the mixed populations to 1:10, we maximize opportunities for interaction by preventing premature disappearance of S. After evolution, we measure changes in killing ability, toxin sensitivity, and resource competitive ability to test for signs of coevolution between K and S strain, as well as between a K host and its virus. Our analyses reveal effects of rapid coevolution between K and S strains: appearance of toxin-resistant mutants that caused initially an increase and then a decrease in killing ability. Changes in killing ability appear to be constrained by a trade-off with resource competitive ability in the absence of sensitive cells. These results show the potential for rapid coevolution at multiple levels of organization in the yeast killer system.

Materials and methods

STRAINS AND MEDIA

We used previously constructed, in the BY background, two isogenic strains: K1 killer and its sensitive counterpart, marked respectively with the resistance to geneticin and

nourseothricin (*ho*::*kan*MX4/*ho*::*nat*MX4) or resistance to hygromycin B (*ho*::*hph*MX4) (Wloch-Salamon et al. 2008). Liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used to grow strains prior to all experiments described below. Low-pH (pH = 4.6) YPD solidified with 2% agar was used for transfers in experimental evolution and competition experiments. YPD agar supplemented with appropriate antibiotics (geneticin: 0.2 mg/ml; nourseothricin: 0.1 mg/ml; hygromycin B: 0.3 mg/ml) was used at one week intervals for special transfers in which killer and sensitive strains were separated and reset to 1:10 ratio and for assaying densities of strains subjected to competition experiments. Assays of the killing ability, the so-called "halo" assays, were done with low-pH YPD supplied with 0.003% MB (methylene blue) and solidified with 2% agar. SC (synthetic complete) medium without uracil was used to select for transformants in cross-infection experiments. In the competitive ability assays of cross-infected killers, SC medium with 1% 5-FOA was used to score colonies with uracil auxotrophy while SC without uracil was used to score colonies with uracil prototrophy.

EXPERIMENTAL COEVOLUTION

Five different experimental treatments were prepared, each represented by three replicate populations (using cells from the same freezer stock per strain): (1) coevolution in which both K and S were allowed to evolve, (2) asymmetric coevolution of K in which only K was allowed to evolve and S was replaced from unevolved freezer stock every week, (3) asymmetric coevolution of S in which only S was allowed to evolve and K was replaced weekly from unevolved freezer stock, (4) control monoculture of K, and (5) control monoculture of S. To start the coevolution (1), stationary phase cultures of K and S were mixed at a ratio of 1:10 (based on measurements of cell densities) and approximately 10^6 cells were spread evenly on 10 ml low-pH YPD agar medium. At every transfer, lasting 24 h, cells were washed off agar surfaces with 10 ml of water and then 1% of it was spread onto fresh agar medium. After every four 24-hour transfers (~25 generations), K and S cells were separated by plating 1% of the wash volume on YPD agar supplemented with an appropriate antibiotic. The plates were incubated for three days and then the coevolving populations were reestablished after measuring the cell density of K and S and mixing them to the initial 1:10 ratio. To start the asymmetric coevolution (2) or (3), stationary-phase cultures of K and S were inoculated onto low-pH YPD agar at a ratio of 1:10 and then 1% transfers were carried out for four days. After the 4th transfer, cells were washed-off and plated on antibiotic agar to select for only K or S. At the same time, "naïve" S or K cells from freezer stocks were plated on relevant antibiotic plates and then used to mix with the evolving counterparts at the beginning of the next week. The two control populations (four and five) consisted of either K or S grown separately. The daily transfers and the three days long incubation on a relevant antibiotic were done in parallel with the symmetric and asymmetric coevolution treatments described above. All experimental populations were transferred for 20 weeks or ~500 generations.

SELECTION FOR SUPERIOR KILLING ABILITY

We also performed an additional experiment in which only K was allowed to evolve. The goal was to test how the cell density of K affects its killing ability and competitive ability. K evolved in the presence of naïve S at three *initial* K:S ratios (1:1, 10:1, and 100:1) all of which were higher than the K:S ratio of 1:10 used in the main experiment. Plates (50 ml) were first seeded with 500 μl of 1000-fold dilution of the stationary phase culture of S (~10^5 cells) and then overlaid with a droplet of 50 μl of the K stationary phase culture containing either ~10^5 (1:1 ratio), ~10^6 (10:1 ratio) or ~10^7 cells (100:1 ratio). The competitors were allowed to interact for 72 hours, producing a clear halo (zone of growth inhibition around the K patch), after which K cells were collected with a sterile loop, suspended in water and their density estimated based on OD reading. S cells from the freezer stock were pregrown to the stationary phase and then K and S cells were adjusted to an appropriate ratio. Five replicate populations at each K:S ratio were transferred this way for 25 transfers consecutively. There were from ~5.5 up to ~8 generations per transfer for the high (100:1) and low (1:1) K density, respectively, leading to ~130–200 generations in total.

KILLING ABILITY ASSAY

Low-pH YPD agar plates (20 ml) supplied with 0.003% MB were inoculated with 200 μl of a 100-fold dilution of the YPD stationary-phase culture of S (~4×10^5 cells per plate). After the plates dried up, 5 μl aliquots of undiluted (~2×10^8 cells/ml) overnight K culture were put on the S cells' lawn as local patches. The size of the zone of growth inhibition (or halo) produced around the K patch was measured manually after 72 hours 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 circle surrounding the halo minus that of the killer patch) divided by the surface area of the killer patch.

TOXIN SENSITIVITY ASSAY

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 S (~10^5 cells). After the patches dried up, 5 μl aliquots of undiluted (~2×10^8 cells/ml) overnight ancestor K cultures were put as small patches onto the S patches. The size of the halo formed around the K patch was measured manually after 72 hours of incubation at 25°C and used as a measure of toxin sensitivity of S (analogously to the killing ability described above).

SPORULATION ASSAY

The sensitive ancestor (one clone), a randomly selected S clone (i.e., carrying the antibiotic-resistance marker associated with the S strain) from each of the three symmetrically and the three asymmetrically coevolving populations from generation 200, as well as a randomly selected S clone from each of the three symmetrically coevolving populations from generation 500 (10 in total) were isolated, sporulated, and subjected to standard tetrad analysis with 10 tetrads per sample.

FITNESS ASSAY

Resource competitive ability (or fitness) of selected isolates was measured in pairwise competition experiments. Every tested K strain was paired with a reference resistant strain (the same in all competitions), every tested S strain with a cured version of ancestral killer (the same in all competitions). To start competition, low-pH YPD agar plates were inoculated with about 2×10^6 cells of a single strain to acclimatize to experimental conditions over 24 of growth. Cells were then washed off with 10 ml of water, mixed in equal volumetric proportion and 10 μl (2×10^5 cells) of the mixture was spread on a new low-pH YPD agar plate. The numbers of both competitors were estimated by plating diluted samples on selective agar media at the start and after 48 hours of competition. Relative competitive ability of the tested strain versus the reference strain was calculated as a ratio of their Malthusian parameters (Lenski et al. 1991).

CROSS-INFECTION OF ANCESTRAL AND EVOLVED VIRUS

Donor K strains were grown in 500 ml liquid YPD medium for three to four days at 30°C. Cells were collected by low speed centrifugation (3000× g), washed with 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 PKE buffer (30 mM Na_2HPO_4 150 mM KCL, 10 mM EDTA, pH = 7.6). Cells were treated with 0.1–1% nonionic detergent (Np40) and incubated for 1 hour at 30°C. Disrupted cells were centrifuged at 4000× *g* for 30 min at 4°C to separate supernatant from the rest of the cell debris. The supernatant was fractionated on a 30% sucrose cushion by centrifugation for 2.5 h at 32,000*g* at 4°C. The resulting pellet with viruses in it was suspended in PKE buffer. The suspension was immediately used for cross-infection. The *pAG60* plasmid with selectable *URA3* gene was used to facilitate cross-infections. The plasmid was mixed with viral supernatant in each transformation. Killer viruses do not carry any selectable marker, hence the phenotype introduced by the plasmid (uracil prototrophy) marked those cells that received the plasmid and thus possibly also the virus. To infect a new host, exponentially growing host cells were collected by low-speed centrifugation (3000× *g*) and washed four times with water. Cells were suspended in 1 M LiAc and immediately collected by centrifugation at 13,000 × g. 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. The resulting mix was incubated for 10 min on ice, followed by 50 min incubation at 30°C and, as a final step, for 10 min at 37°C. The cells were collected by centrifugation for 30 s at 8000 × *g*, suspended in YPD and immediately spread on SC-uracil plates. After three days of incubation, colonies were picked, grown up and stored at –80°C.

STATISTICAL ANALYSES

We used pairwise comparisons based on two-sample *t-tests* (with unequal variance) to test for significance of phenotypic differences. To test time differences in the evolution of resistance between treatments, we fitted logistic models, estimated the time when 50% of the maximum phenotype was reached, and used two-sample *t*-tests. The density-dependent effect of killing ability was tested by a one-way ANOVA. A two-way ANOVA was used to test for the effect on competitive ability exerted by the interaction between K:S ratio and presence/absence of sensitive cells. To test for the trade-off between killing ability and resource competitive ability, we used the Pearson's correlation coefficient. The evolved versus ancestral virus effect of killing ability was tested in one-way ANOVA. A two-way ANOVA was used to test for the effect of host and virus on killing ability from the cross-infection with foreign/own viruses.

Results

We evolved populations of two strains of *S. cerevisiae* (isogenic except for an antibiotic-resistance marker), one (K) carrying a cytoplasmic toxin-producing killer virus K1, the other (S) without the virus and hence sensitive to the toxin. Experimental populations were propagated on YPD low-pH agar medium for 500 generations under three different treatments: symmetric coevolution, where both K and S were transferred and allowed to evolve in mixed populations, asymmetric coevolution, where only K or S was transferred and the other strain was weekly replaced from the same "unevolved" freezer stock for all replicates, and control monoculture populations of K or S. The K:S ratio in the coevolution and asymmetric coevolution treatments were weekly reset by passing the two strains through relevant selective media and mixing them again at 1:10 ratio to ensure stable opportunity for interaction, that is, prevent K from rapidly overtaking whole populations (Wloch-Salamon et al. 2008). Figure S1 shows the frequency of evolving K and S at the end of each week, that is, before their ratio was reset to 1:10. The rapid increase of K, and the corresponding decline of S, in the first week (~25 generations) reflects the competitive superiority of K under these conditions. The invasion of K and reduction of S declined in all

treatments after the first week. The final density of K was lower under symmetrical than asymmetrical evolution (Fig. S1A vs. B; *t*-test on final density of K: $t = -3.01$, $df = 4$, 2-tailed $P = 0.039$). The final density of S did not differ between the two treatments (Fig. S1A vs. C; $t = 1.76$, $df = 4$, 2-tailed $P = 0.152$).

EVOLUTION OF KILLING ABILITY AND TOXIN SENSITIVITY

To measure the killing ability of K and toxin sensitivity of S during experimental coevolution of five treatments, 20 clones of each strain from each of the three replicate populations per treatment were isolated at each 100-generation interval (summing up to 1840 clones: six strain-treatment combinations × three replicate populations × five time points × 20 clones, together with 20 K and 20 S ancestral clones) and assayed using the halo test (see Methods). Figure 1 shows changes in average killing ability and toxin sensitivity across treatments and time. To compare trajectories across conditions, we used two-sample *t*-tests (with unequal variance) to test for significant changes relative to the ancestor. In the absence of S, killing ability slightly increased and then decreased. The evolutionary dynamics was more conspicuous in the mixed populations: K evolved clearly higher killing abilities when S was not allowed to evolve. When S was allowed to evolve, K initially evolved high killing ability but ended with the lowest killing ability (Fig. 1A). Changes in toxin sensitivity of S were less complex (Fig. 1B). In the absence of K, sensitivity increased monotonically—presumably as a correlate of increased resource competitive ability (see below). When K was present, toxin sensitivity decreased in both mixed treatments to similar final levels, although the decrease happened much earlier when both K and S were allowed to evolve (2-tailed $P < 0.01$ for *t*-tests comparing these two treatments at 100, 200, 300, and 400 generations). Interestingly, the initially faster decline in toxin sensitivity of S under coevolution conditions coincided with the initial increase in killing ability of K under these conditions, suggesting that these changes were triggered reciprocally by the coevolving strains. Note that the parallel dynamics among the three replicate populations may be partly due to shared standing genetic variation present at the start, since replicate populations were started with cells from the same freezer stock.

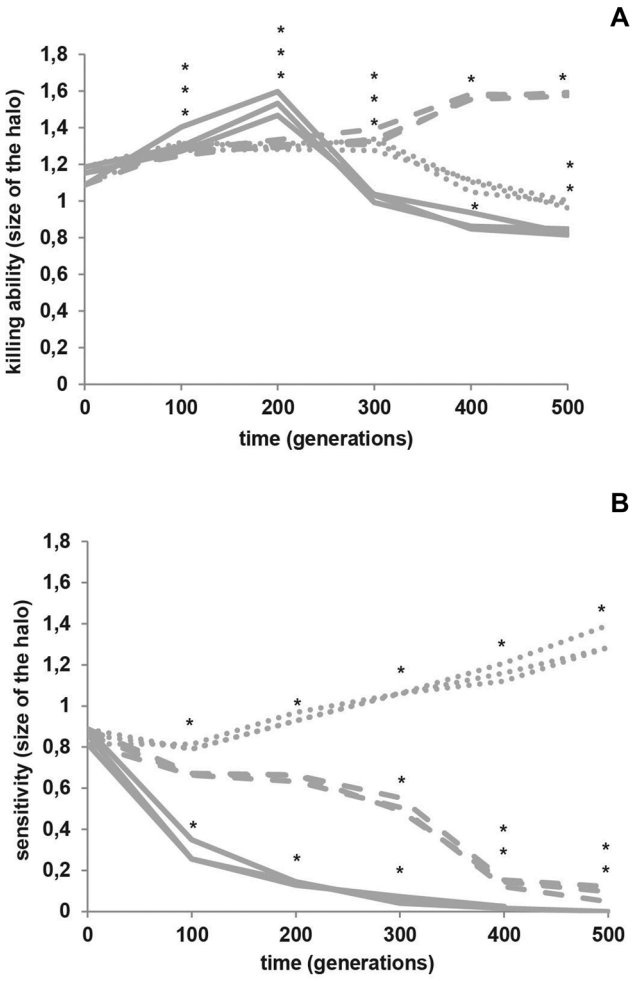

Figure 1. Killing ability of K (A) and toxin sensitivity of S (B) under the three experimental conditions (coevolution of both K and S—solid line; asymmetric coevolution of K or S—dashed line; monoculture control of K or S—dotted line). Both killing ability and toxin sensitivity are measured by the relative zone of growth inhibition in confrontations of K and S cells under standardized conditions (see Methods). Estimates are derived from measurements of 20 clones from each time point and replicate population. Asterisks indicate significant changes relative to the ancestor based on pairwise comparisons corrected for multiple testing (Rice 1989, using $P < 0.05$).

EVOLUTION OF TOXIN RESISTANCE

The rapid loss of toxin sensitivity in the coevolving populations (Fig. 1B) suggests that it arose early by a single mutation. To examine this possibility, we selected at random 20 S clones per time point and treatment and plotted the frequency of clones showing the complete loss of toxin sensitivity (i.e., forming no halo when confronted with the ancestral K strain; Fig. 2A). All tested S clones from the control populations were sensitive to the toxin, while the frequency of nonsensitive clones in the mixed populations was a mirror image of the populations' average sensitivity to the toxin (see Fig. 1B). Furthermore, none of the tested clones showed intermediate toxin sensitivity. These findings confirm that the loss of sensitivity indeed followed a simple scenario: the occurrence of a completely resistant strain and its rapid rise in frequency. This increase was faster in cultures in which K was allowed to coevolve than when it was replaced every week with the ancestral K strain (comparing estimates of the time when reaching 50% frequency using a logistic model: $t = -14.88$, $df = 4$, two-tailed $P < 0.0001$).

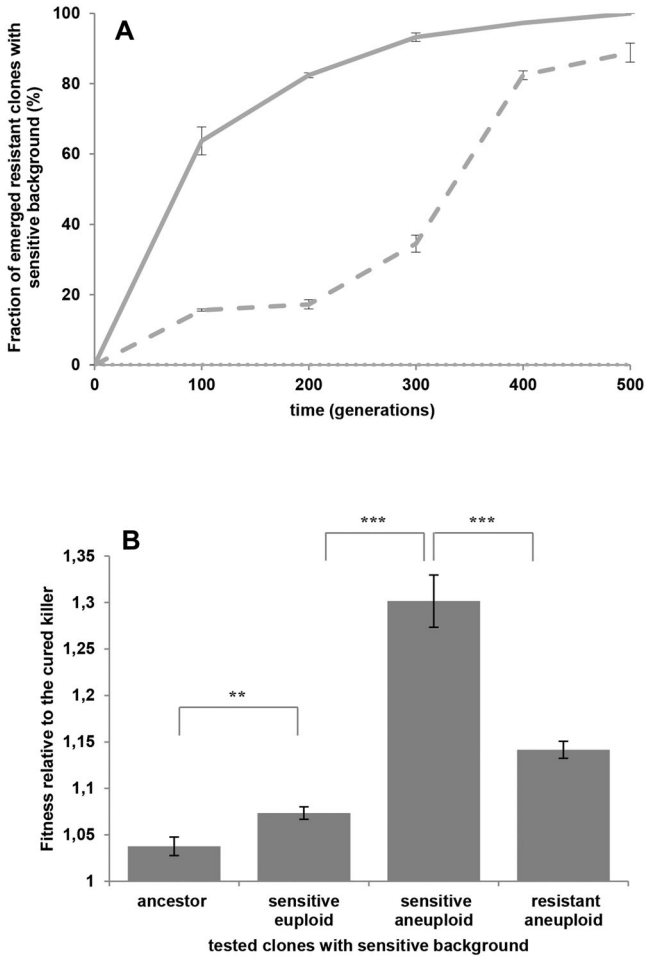

Figure 2. Evolution of toxin resistance. (A) Ascendance of the de novo evolved and fully toxin-resistant mutants in the S background; coevolution of both K and S (solid line), asymmetric coevolution of S only (dashed line), and monoculture control of S (dotted line, showing no resistant mutants). Errors bars represent standard errors of the mean based on estimates for three replicate populations per treatment. (B) Changes in competitive fitness relative to K cured from its virus. "Ancestor" is the sensitive ancestral diploid, "sensitive euploid" and "sensitive aneuploid" are presumed euploid and aneuploid isolates from generation 200, "resistant aneuploid" are presumed aneuploid isolates from generation 500. Aneuploidy is inferred by sporulating isolates and testing haploid spores for toxin resistance. Error bars represent standard errors of the mean based on 10 replicate assays. Asterisks indicate significant differences in pairwise comparison (*P < 0.05, **P < 0.01, ***P < 0.0001).

To test the hypothesis that resistance involved a single mutation, we first sporulated the ancestral S clone and nine S clones from two treatments and different time points: a toxin-sensitive clone from each of the three coevolving and three asymmetrically coevolving populations after 200 generations when resistance was polymorphic, and a fully resistant clone from the final time point of each of the three coevolving populations (500 generations). At least 10 tetrads from each clone were tested for the pattern of segregation of the resistant phenotype. As expected, the ancestral S clone always yielded four toxin-sensitive haploid spores. However, the 500-generation resistant clones showed an unexpected segregation pattern: two of the four spores in the tetrad were always unviable and the other two fully resistant. The S clones from generation 200 showed a polymorphic pattern: 25 yielded four sensitive spores while 35 had two viable sensitive spores and two unviable spores. Together these findings suggest that the evolution of toxin resistance was a two-step process. A possible scenario involves the loss of a copy of a single chromosome (causing the 2:2 segregation of viable and nonviable spores), followed by a mutation causing toxin resistance on the haploid chromosome. The latter suggests that the mutation is recessive and explains why no tetrads were found with different segregation patterns.

To better understand the role of natural selection in the hypothesized scenario, we performed competition experiments involving an ancestral clone, euploid sensitive clones, presumed aneuploid sensitive clones, and fully resistant aneuploid clones (Fig. 2B). Euploid sensitive clones showed a small but significant increase in competitive ability, while the presumed aneuploid sensitive clones from the same time point showed a remarkably large competitive benefit of ~30%, which explains the rapid rise in frequency of these aneuploidy mutants. The fully resistant clones had lower competitive ability than the sensitive aneuploid clones but still higher than the ancestral and the 200-generation sensitive and fully euploid clones. These results support an important role for natural selection during the two-step evolution of toxin-resistant mutants. First, the loss of a chromosome provided a large resource-competitive benefit allowing the rapid spread of this genotype. This prepared the stage for a recessive resistant mutation that occurred on the single-copy chromosome and provided a further net fitness benefit resulting from an interference-competitive benefit in the presence of K which more than compensated the associated decrease in resource-competitive ability.

We finally asked whether the raise of resistant mutants would be affected by the frequency of K–S interactions. We addressed this question by employing additional short-term coevolution experiments using 1:10, as before, 1:1 and 1:100 K:S to vary the frequency of K:S encounters. Three replicate populations per treatment were used and K:S ratios were not periodically reset but allowed to change. After evolution single clones were tested for toxin sensitivity (Fig. S2). We found that at equal frequencies of both competitors, resistance could not develop due to the rapid elimination of S cells (which were lost after two transfers). For the 1:100 ratio, resistant mutants emerged, but they appeared and became fixed later than in the 1:10 ratio populations, indicating that the evolution of toxin resistance in S cells is sensitive to the frequency of interactions with K cells, and presumably most

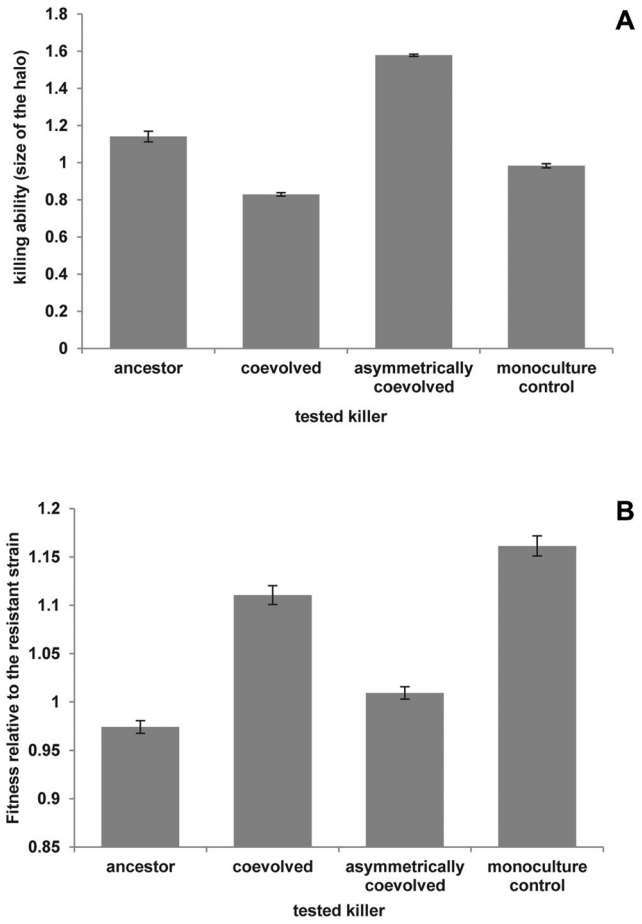

Figure 3. Average killing ability (A) and relative fitness (B) of the ancestral and 500-generations evolved K clones. Strains derived from coevolution of both K and S, asymmetric coevolution of K (with replacement of S) and control evolution (i.e., monoculture of K). Fitness of K was measured in the absence of sensitive cells. Error bars represent standard errors of the mean based on three clones of the ancestor and three replicate evolved strains per condition.

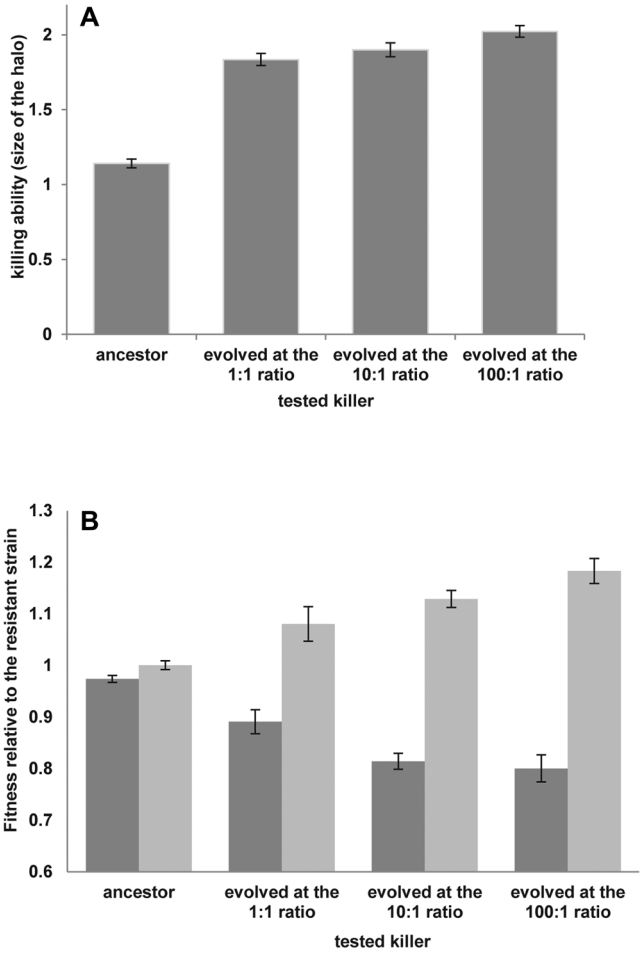

Figure 4. Selection for increased killing ability at varying K density. K strains were grown together with S for ~130–200 generations at three initial K:S density ratios (1:1, 10:1, and 100:1). (A) Killing ability of ancestor and evolved K strains. (B) Competitive fitness of K strains relative to a toxin-resistant strain. Assays were conducted in the absence (dark gray) and in the presence (light gray) of ancestral S cells. Error bars represent standard errors of the mean based on five replicate assays.

rapid when encounters of K and nonkilled S cells is maximal (1:10 instead of 1:1).

TEST OF A TRADE-OFF BETWEEN FITNESS AND KILLING ABILITY

To examine the relative importance of resource versus interference competitive ability and possible trade-offs between them, we compared evolved changes measured in both competitive abilities. All pairwise comparisons of mean killing ability (Fig. 3A) and mean fitness (Fig. 3B) between the ancestor and the final evolved clones showed significant differences (two-tailed $P < 0.0001$ for killing ability and $P < 0.01$ for competitive ability using *t*-tests). Inspection of Fig. 3 yields two conclusions. First, the moderate decline in killing ability observed for the K control populations (evolved in the absence of S) was associated with the largest increase in competitive ability, suggesting that evolution of these populations was driven by resource competition.4 Second, the impressive improvement in competitive ability in the coevolving populations was unsustainable. A large decrease in killing ability ensued and it was associated with a substantial (~15%) increase in competitive ability. Together, these changes suggest that a trade-off between killing ability and competitive ability exists: evolution leads either to improved resource competition (in the absence of S) or to improved interference competitors (in the presence of S), but not both.

We made an attempt to select for even higher killing abilities by increasing the initial density of K relative to S (1:1, 10:1 and 100:1, instead of the original 1:10). The K cells were concentrated in a small patch surrounded by a lawn of ancestral S cells. Cells from the edge of the K patch were used to initiate the next transfer

(25 cycles, equivalent to ~130–200 generations) in five replicate populations per treatment. Despite the shorter evolution, killing abilities increased beyond those observed in the original asymmetrically evolved K populations (Fig. A). In addition, initial K density had a significantly positive effect on final killing ability (one-way ANOVA: $F = 67.29$; $dfs = 3, 14$; $P < 0.0001$), possibly due to associated increases in the mutation supply of K and hence its ability to evolve.

We then measured competitive ability of evolved K strains under two conditions: (i) in the absence of S when only resource competition affects fitness, and (ii) in the presence of S when resource and interference competition are both important. Figure 4B shows that, in absence of S, there was a significant reduction in resource competitive ability accompanied by an increase in killing ability. However, when S was present, the evolved K strains showed higher competitive ability (two-tailed $P < 0.01$ using pairwise comparisons) while the ancestor did not. Moreover, the larger the benefit in the presence of S the larger the cost in the absence of S (interaction between K:S ratio and presence of S: $F = 11.85$; $dfs = 1,2$; $P < 0.0001$).

Finally, to formally test for a trade-off between killing ability and resource competitive ability, we pooled all estimates gathered for the ancestral and evolved K strains (using mean estimates per treatment). As shown in Fig. 5, these data support the existence of a trade-off across evolutionary treatments (Pearson's $r = –0.92$, $n = 7$, $P = 0.003$).

HOST–VIRUS COADAPTATION

Finally, we tested whether coevolution happened not only between K and S strains, but also between K hosts and their killer viruses. We isolated viruses from the ancestor and clones from four evolved K strains (symmetrically coevolved, asymmetrically coevolved, evolved in a monoculture, and coevolved under the highest K:S density ratio 100:1). Virus-cured versions of the four evolved hosts were reinfected with their own viruses and that of the ancestor. The ancestral K strain was reintroduced with own virus and the virus derived from the evolved at the 100:1 ratio K. All ten host–virus combinations were then assayed for killing ability (Fig. 6). Comparative analysis of the effect of evolved versus ancestral virus in the four evolved K strains demonstrated that carrying original, evolved virus has a substantial contribution into their final killing ability (one-way ANOVA: $F = 13.30$; dfs = 1, 28; $P = 0.001$). Furthermore, analysis of variance of the ancestral and evolved at the 100:1 ratio Ks, for which cross-infection combinations were constructed indicates that changes in both host and virus contributed to the changes in killing ability of evolved strain (Table 1). The highly significant interaction term points to negative effect of new and beneficial of old combinations. This dependence of the effect of changes in host and virus on each other strongly suggests that they have been reciprocally triggered, which is the hallmark of coevolution.

Table 1. Two-way ANOVA on the effect of host and virus on killing ability from the cross-infection of killer viruses between ancestor and 100:1 K:S ratio-evolved K.

Effect	*df*	*MS*	*F*	*P*
Host	1	0.578	110.41	<0.0001
Virus	1	1.277	243.82	<0.0001
Host × virus	1	0.083	15.82	0.004
Residual	8	0.007		

Discussion

We demonstrate the rapid and parallel coevolution in mixed populations of two strains of *S. cerevisiae*, one carrying K1 killer virus encoding an anti-competitor toxin (K), the other an isogenic toxin-sensitive strain without virus (S). Coevolution proceeded at two distinct levels: between K and S strains and between the yeast host and its killer virus. At the level of interactions between strains, toxin-resistant mutants appeared quickly within the S strain. The rise of resistance initially accelerated the evolution of an increased killing ability of the K strain but later—when the frequency of sensitive cells dropped below a critical value—promoted selection for a decreased killing ability. We maintained experimental populations under three conditions, where both K and S evolved, where only one strain evolved while the other was replenished from the freezer stock, and where the competitor strain was absent altogether. Comparing results obtained under these treatments, we could determine that some evolutionary changes in K and S were reciprocal and thus demonstrate true coevolution.

Coevolution also occurred at the level of host–virus interactions within K. It was detected after swapping the virus between ancestral and evolved strains, which showed that only combinations of coevolved partners exhibited considerable improvement in killing ability. The rapid coevolution that we observed between yeast host and killer virus suggests that also in nature coadaptation may happen at short time scales. It implies that once a killer virus enters a new host (e.g., via a sexual cross), where it presumably incurs an initial fitness cost (Wloch-Salamon et al. 2008), the association may rapidly stabilize as a result of coadaptation.

Signatures of coevolution have been observed in many organisms, including bacteria and bacteriophages (Buckling and Rainey 2002; Forde et al. 2008), bacteria and archaea (Hillesland and Stahl 2010), beetles and microsporidia (Bérénos et al. 2011), figs and pollinating wasps (Cook and Rasplus 2003), although often without information about the dynamics of the process and whether adaptation was reciprocal. Genuine coevolutionary

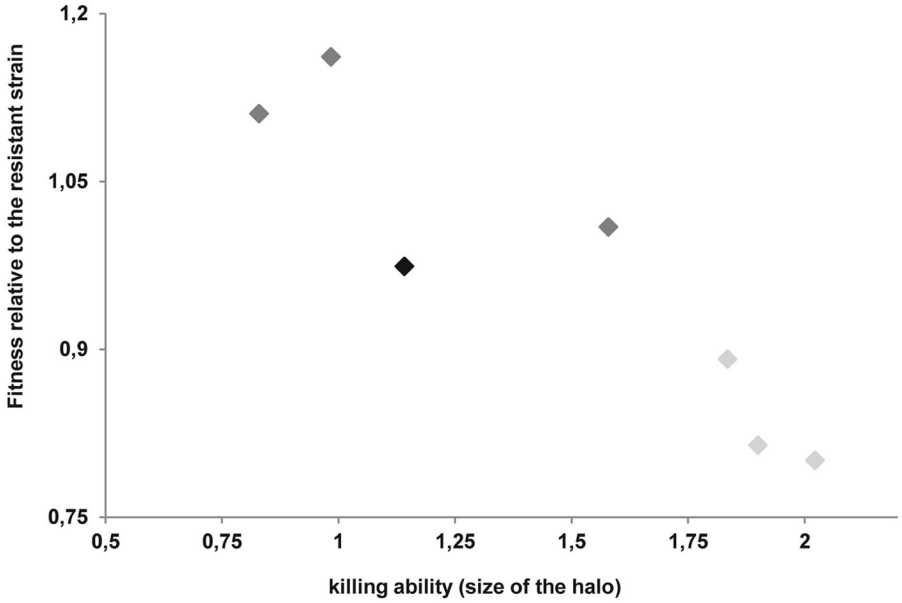

Figure 5. Relationship between the mean killing ability and relative fitness of the ancestral (black) and six evolved K strains, including the three treatments of the main experiment (coevolution, asymmetric coevolution, and monoculture control; dark gray) and the additional asymmetric K evolution at three K:S ratios (light gray). Killing ability and fitness are negatively correlated ($r = -0.92$, $n = 7$, $P = 0.003$).

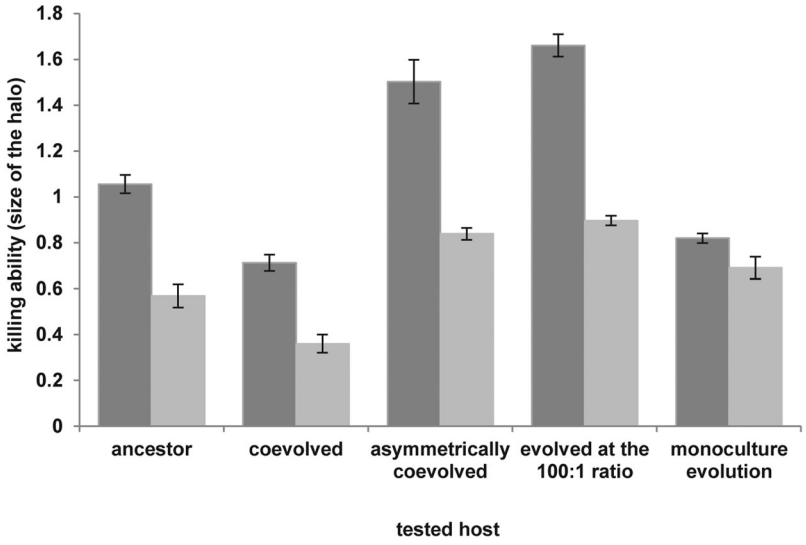

Figure 6. Killing ability of K strains that either carry their own virus (dark gray) or a new virus (light gray). The strains involved were derived from different evolution treatments and include the ancestor. New virus was that of the K ancestor for the four evolved strains, and the virus from the K strain evolved at the 100:1 ratio for the ancestor. Error bars represent standard errors of the mean based on the mean estimates for three replicates.

responses between viruses and their hosts were demonstrated for bacteria (Lenski 1988; Buckling and Rainey 2002; Forde et al. 2004; Paterson et al. 2010), but not for yeast. A crucial difference between our study and the bacteria-phage experiments is that the latter involve mostly one-sided antagonistic interactions. Any decrease of bacterial fitness resulting from evolution of the phage, does not affect phage fitness as much as it does affect the killer virus in yeast, because bacteriophages can escape their host and infect new hosts, but this is much less likely for yeast killer viruses (Stenseth and Smith 1984).

A somewhat related study to ours addressed coadaptation between the nuclear and mitochondrial genome in experimentally evolved yeast populations (Zeyl et al. 2005). By swapping mitochondria between ancestral and evolved cells, it was shown that fitness of the evolved strains was aided by mutations in both genomes. However, while we found strong interactions between

evolved host and virus at the level of killing ability (Fig. 6), the fitness effects of evolved nuclei and mitochondria were more or less additive. Signs of addiction have been reported for a bacterium-plasmid association. After extended propagation of bacteria carrying a plasmid with an antibiotic resistance gene, the evolved plasmid enhanced fitness of the evolved host whereas the original plasmid reduced fitness of the ancestral strain (Bouma and Lenski 1988).

A second remarkable result of our study is the strong support we observe for a trade-off between killing ability and resource competitive ability (i.e., in the absence of sensitive cells; see Fig. 5). Trade-offs between fitness components are considered crucial for understanding the existing differences between species, in particular the divergence between alternative competitive strategies (Stearns 1989; Duffy et al. 2007). Fitness trade-offs were previously demonstrated for antibiotic resistance (Andersson and Hughes 2010; MacLean et al. 2010) and virulence (via toxin production) (Cascales et al. 2007; Berenos et al. 2009). We showed that the ability of K strains to kill correlated negatively with their ability to compete for resources with strains resistant to the toxin. An enhanced ability to kill was apparently costly and this stimulated specialisation towards either increased killing ability under conditions making it helpful (e.g., low dispersal, frequent interactions with sensitive cells and high local density of toxin producers) or increased resource competitive ability (under opposite conditions), but not evolution of generalists that would be good in both (Brockhurst and Koskella 2013).

One of the most striking findings of our study was the rapid emergence and invasion of toxin resistant mutants derived from the sensitive strain. Segregation analyses of genotypes from various time points together with resistance and fitness measurements suggest that resistance evolved in two subsequent steps despite its rapid appearance. First, mutant cells lacking one chromosome (2n-1) arose and invaded driven by a large (~30%) resource competitive advantage. Then, these aneuploidy mutants acquired a mutation causing toxin resistance that was most likely recessive and located on the single-copy chromosome (because all resistant genotypes tested showed segregation of resistant and inviable haploids). Rapid invasion of aneuploid genotypes has previously been observed in yeast (Pavelka et al. 2010; Sunshine et al. 2015). In principle, alteration of gene dosage and the uncovering of beneficial recessive alleles (present in the ancestral diploid strain) may explain the fitness benefit of aneuploids. The large fitness benefit associated with the loss of a chromosome suggested that the S monoculture populations may harbour similar aneuploidy mutants. We tested this by performing similar tetrad analyses of clones isolated from these populations at 200 and 500 generations, but here the pattern of segregation was more variable (ranging from four to only one viable spore per tetrad). This confirmed that chromosome loss also here likely played a role, but that mechanisms were more diverse than the loss of a single chromosome.

In sum, our results demonstrate the potential for rapid multilevel coevolution in the yeast killer system. Within 500 generations of laboratory evolution, we observed two processes: coevolution between yeast cells and their cytoplasmic K1 killer virus particles, as well as between cells hosting viruses (killers) and those deprived of them (nonkillers, initially sensitive with resistant variants emerging among them). The two processes were not independent, since changes in killing and resource competitive ability were driven by interactions between killer and nonkiller cells, while they arose from, and had consequences for, interactions between the yeast host and its killer virus (Bolnick et al. 2011). However, the exact causal relationship between both levels of interaction is yet to be resolved. This will require additional experimental work, preferably laboratory evolution experiments where the opportunity of components to evolve is controlled.

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DATA ARCHIVING

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.gk1hk

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Average frequency (cell density per ml) of killer (K – solid line) and sensitive (S – dashed line) cells in mixed populations during the 500 generations of evolution under three conditions: (A) coevolution of both K and S, (B) evolution of K only (with weekly replacement of S from "unevolved" freezer stock), and (C) evolution of S only (with weekly replacement of K from "unevolved" freezer stock).

Figure S2. Frequency-dependent evolution of toxin resistance.