Role of anti-competitor toxins in the origin and maintenance of diversity in *Saccharomyces* yeast microbial populations

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Chapter 1

General introduction

Experimental evolution

Experimental evolution has become a common tool in evolutionary biology and microbial ecology. Its standard approach is to use replicate populations and controlled conditions (environmental, demographic, and genetic). It has been widely used to address a variety of questions and problems such as estimating the fitness effects of spontaneous mutations (Orr 2009; Matsuba et al. 2013; Wang et al. 2013), adaptation to different environmental regimes (Bennett and Lenski 1993; Kolss et al. 2009; Dhar et al. 2011), social interactions between and within species (West et al. 2006; Queller and Strassmann 2013), and detecting ecological and evolutionary trade-offs (Rose 1984; Fry 2003; Roff and Fairbairn 2007). Much of the previous work has been done with bacteria but it is now recognized that using yeast provides a number of important advantages. For instance, an important and typically eukaryotic trait is the ability to recombine the genome through sexual reproduction. Fascinating experimental evolution studies using yeast range from the focus on evolution of multicellularity (Ratcliff et al. 2012), the dynamics and reproducibility of adaptation (Lang et al. 2013), through to nuclear-mitochodria coevolution (Zeyl et al. 2005). Moreover, an abundance of genes that have homologs in humans provides an opportunity to analyse processes related to human diseases in evolutionary terms. Yeast has helped to make important insights into the molecular mechanisms involved in the eukaryotic genome evolution (Dujon 2006).

Furthermore, an essential prerequisite for experimental evolution in yeast has also been fulfilled: a well-equipped experimental toolbox. A full genome sequence has been available since 1996, and an especially large number of protocols for phenotypic and genomic analyses has established. Therefore, yeast is now recognized as an indispensable eukaryotic model system for the study of eukaryotic genetics and cell biology (Jasmin et al. 2012; Jasmin and Zeyl 2012) and mechanism of social interactions (Datta et al. 2013; Van Dyken et al. 2013).

"Killer yeast"

This thesis concentrates on the so-called killer phenotype of yeast which is coded by RNA-based viruses. Viruses are the most abundant biological entities on Earth and can be found in almost every organism and physical habitat. Viruses are capable of infecting almost any organism, including yeast. Viral elements that can be found in yeast cells include retro- and double-stranded RNA (dsRNA) viruses (Wickner 1989; Kirchner et al. 1995; Bushman 2003; Zhu et al. 2003). Most of these viruses are non-infectious; hence they are often referred to as ''virus-like-particles'' (VLPs). The field of yeast virology has begun with the detection of viral elements within the genus *Saccharomyces* (Bevan and Makower 1963). In yeast, dsRNA viruses produce low-

molecular mass proteins or glycoproteins which act, after secretion by the yeast cell, as toxins against yeast cells that do not carry these viruses. Since their discovery, it has been repeatedly reported that some yeast strains are capable of producing and secreting exotoxins that kill other strains of the same or closely related species or genera (Schmitt and Breinig 2006). They have been dubbed "killer yeasts" and have been regarded as endosymbiotic partners of their yeast hosts (McBride et al. 2008). It has become gradually evident that killer strains occur not only within Saccharomyces, but also in a wide range of other yeasts and fungi including *Candida, Cryptococcus, Pichia, Ustilago, Torulopsis, Zygosaccharomyces, Hansenula, Williopsis, Debaryomyces, Hanseniaspora,and Kluyveromyces* (Zorg et al. 1988; Radler et al. 1993; Park et al. 1996; Schmitt and Breinig 2002).

The killer phenotype in *Saccharomyces cerevisiae* is strictly associated with the presence of dsRNA viruses belonging to the *Totiviridae* family, the best characterised class of mycoviruses (Magliani et al. 1997; Marquina et al. 2002; McBride et al. 2013). The killer phenotype requires the presence of two separately encapsulated dsRNA viruses: an L-A helper virus and a toxin-coding M-satellite virus. The LA subunit of 4.6 kb, which encodes the major capsid protein (Gag) and the viral RNA-dependent RNA polymerase (Pol), is an autonomously replicating virus and is commonly found in fungi (Icho and Wickner 1989; Ghabrial 1998). Its genome has been extensively studied. The M subunit consists of 1.6-2.1 kb; it is a satellite virus and contains a set of genes responsible for the production of a toxin and an associated immunity factor. The presence of both subunits together is required to make the toxin active which determines the killer phenotype (Fig.1).

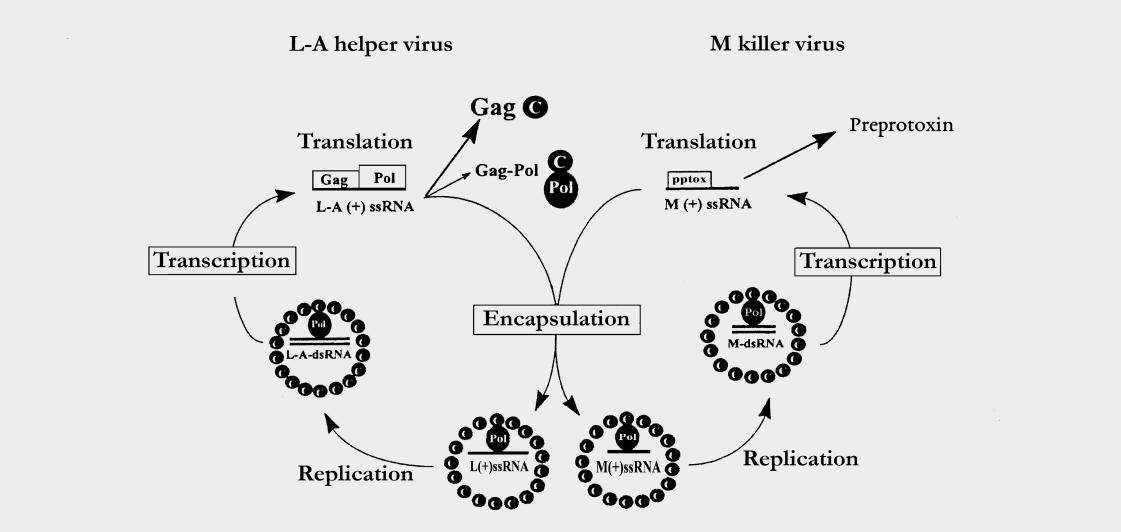

Figure 1. Replication of the LA and M killer viruses. Both subunits compete for viral proteins (Gag and Gag-Pol) encoded by the LA subunit. These proteins are required for the viral assembly and transmission into the new host. Shown are viral replication and synthesis on the double-stranded RNA template (Schmitt and Breinig 2006).

To date, three principal *Saccharomyces* viruses (ScV-M1, ScV-M2 and ScV-M28) have been characterized. They code for K1, K2 and K28 killer phenotypes, respectively. In all killers, killing proceeds via

freely released toxins in a two-step receptor-mediated process (Fig.2). The first stage does not require an input of energy, and includes quick binding of heterodimeric protein to receptors that are present in the cell wall of sensitive cells; the main receptor for toxins K1 and K2 is a β-1,6-D-glucan, while for toxin K28 it is a α-1,3-mannoprotein. The second stage is energy-dependent and involves the translocation of toxins K1 or K2 to the cytoplasmic membrane followed by interaction with its specific receptor. The consequence of binding of the toxin to the cell membrane is a series of physiological changes that result in the death of sensitive cells. Initially, the amino acid proton gradient collapses, followed by leakage of potassium ions, release of ATP, reduction of metabolite levels and damage of the cell-membrane pH gradient (Tipper and Schmitt 1991; Ahmed et al. 1999; Flegelová et al. 2002; Rodríguez-Cousiño et al. 2011). The mechanism of killing by the K28 toxin differs significantly. While K1 and K2 act on the surface of cytoplasmic membrane, K28 enters the cytosol by endocytosis (Schmitt et al. 1996; Eisfeld et al. 2000). After binding to a receptor, it travels through the Golgi complex and endoplasmic reticulum to the cytosol, where it sends a signal to the nucleus. This causes blocking of DNA synthesis and cell cycle arrest at the early S phase of the cell cycle, contributing to the loss of cell viability (Schmitt and Breinig 2002, 2006; Rodríguez-Cousiño et al. 2011). The process of endocytosis and retro-translocation of proteins and toxins into the cytosol is commonly observed in plants and bacteria (Lord and Roberts 1998; Tsai et al. 2002).

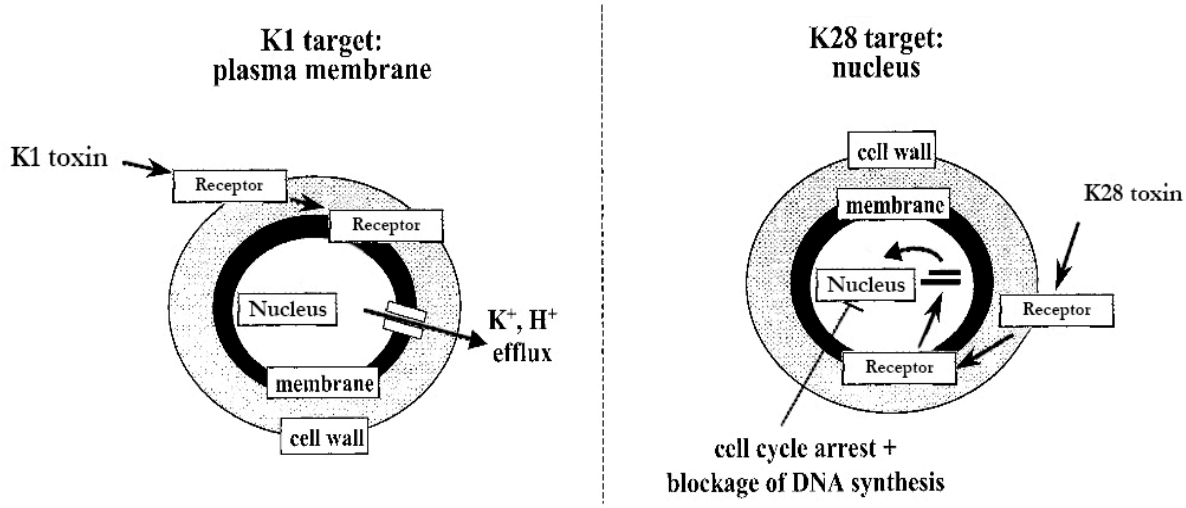

Figure 2. The mode of K1 and K28 viral toxin action in *Saccharomyces* yeast. Sensitive cells are killed in a two-step receptor-mediated process. Toxins bind to the cell wall and cytoplasmic membrane receptors, leading to the disruption of cytoplasmic membrane function (K1) or inhibiting DNA synthesis (K28) (Schmitt and Breinig 2002).

In contrast to the horizontal transmission of the majority of pathogenic plant and animal RNA viruses, 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. Except for the dsRNA viruses, the killing phenotype can also occur via linear dsDNA plasmids (Worsham and Bolen 1990; Hayman and Bolen 1991) or nuclear genes (Suzuki and Nikkuni 1994; Hodgson et al. 1995). In all killer yeasts, production of the toxin is closely associated with the production of an immunity component. In the killer yeast the factor responsible for immunity to the toxin is still unidentified. However, it has been suggested that the toxin precursor might have a role in immunity by acting as a competitive inhibitor of the mature toxin and saturating or eliminating the plasma membrane receptor which normally confers the toxicity (Bussey et al. 1983; Tipper and Schmitt 1991).

Killer yeast strains have been frequently found in natural materials, such as fruits, mushrooms, soil, and decaying plant material. They are believed to play a significant role in ecology as they shape the composition of yeast communities (Ganter and Starmer 1992; Pintar and Starmer 2003). Killer toxins have also found numerous applications, such as in the food fermentation industry, where they are used to control contaminants (Javadekar et al. 1995), in the development of novel antimycotics to treat fungal infections (Buzzini and Martini 2001), and in the field of heterologous protein production and secretion (Giga-Hama and Kumagai 1999).

Experimental assays of the killer phenotype

Measurements of the killing ability of the killer yeast strains rely on estimating the rate at which they eliminate cells of a sensitive strain and are particularly important to understand the ecological and evolutionary role of toxin production. Different methods have been recently used to estimate the killing rate of toxin-producing cells. The “Halo method” estimates the size of the zone of no growth surrounding a patch of toxin producers growing on agar medium overlaid with a layer of sensitive cells (de Ullivarri et al. 2011; Santos et al. 2011; Maqueda et al. 2012; Mehlomakulu et al. 2014). An alternative method, the “Serial-Dilution method”, estimates the maximum dilution of droplets of spent liquid culture from a toxin producer that still inhibits the growth of sensitive cells growing on agar (Schoustra et al. 2012). There are also methods for estimating the rate of killing in liquid cultures of sensitive cells confronted with toxin-producing strains or their products (Alfenore et al. 2003; Novotná et al. 2004; İzgü et al. 2006). For killer yeasts, the “Halo method” (Fig.3) has been most commonly used to estimate killing rates and this method is able to discriminate between different toxins (Kishida et al. 1996; Wloch-Salamon et al. 2008; McBride et al. 2013).

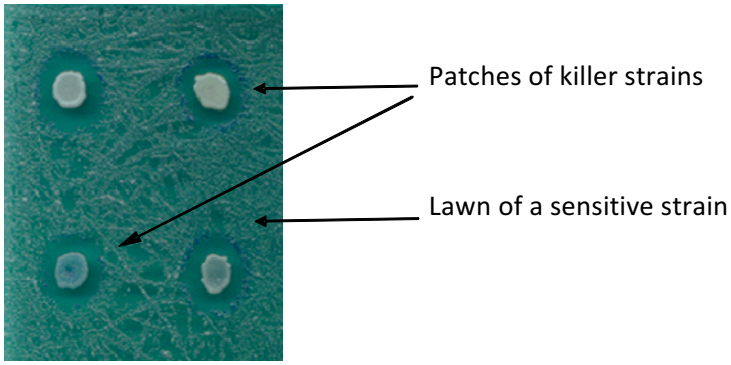

Figure 3. Identification of the killer phenotype of *Saccharomyces*. Agar plate seeded with cells of a sensitive strain and then overlaid with dense patches of cells of tester killer strains. After incubation, a zone of growth inhibition around the patches of the killer strains can be measured to compare killing rates of different killer strains.

What explains the diversity of microbial communities

One of the central questions addressed in this thesis involves understanding whether anti-compeitor toxins can explain the occurrence and maintenance of microbial diversity.

Resource* versus *interference competition

Recent years witnessed growing interest in social interactions of microbial populations both among members of the same and different species (genotypes) (Foster 2011). Such interactions may be cooperative (altruistic) if one genotype provides benefits to another by secreting compounds such as enzymes that break down complex proteins into nutrient sources, which results in promoting growth and thereby increases fitness (West et al. 2006; Foster 2011). Interactions via production and secretion of toxins are called spite, because toxin producers accept carrying a fitness cost if this helps to impose larger cost on the fitness of non-producers. Competition for limiting resources between organisms which share the same environment is one of the most important driving forces in evolution. Interference competition via anti-competitor toxin production and secretion is widespread in microbial populations, including bacteria (Chao and Levin 1981; Dykes 1995; Riley 1998) and yeasts (Tipper and Bostian 1984; Starmer et al. 1987; Jacobs and Van Vuuren 1991; Abranches et al. 1997; Schmitt and Breinig 2002). The exertion of toxic compounds can also be found in numerous other species, such as Paramecium (Grun 1976), metazoan sponges (Thompson et al. 1985), and plants (Rice 1984; Callaway and Aschehoug 2000). Toxin production carries a cost to its producer, thereby reducing its (resource) competitive ability relative to a non-producer. Therefore, a trade-off

between killing and resource competitive abilities is expected to occur if there are non-producing competitors in the environment.

Such a trade-off may lead to specialisation (investment either in killing or resource competition) and prevents the evolution of generalists which would be a jack in both strategies, but a master in neither. A trade-off between benefits in resource competition and interference competition is a likely explanation of the coexistence of different competitors (Czárán and Hoekstra 2003; Brown et al. 2009; Hibbing et al. 2010). Toxin-producers invade populations of sensitive cells and this enables them to get access to the available resources in these populations. However, toxin-sensitive individuals may evolve toxin resistance, despite possible fitness costs, which improves their competitive abilities and allows surviving. This may result in cyclical coexistence (through negative frequency-dependent selection) of toxin producer, sensitive, and resistant competitors, in which producers outcompete sensitive, resistant outcompete toxin producers, and sensitive outcompete resistant due to the highest resource competitive abilities (Kerr et al. 2002; Czárán and Hoekstra 2003).

Cost of toxin production

Toxins are typically targeted against sensitive species of the same or related genera. Two types of bacterial toxins (bacteriocins) have been studied most intensively: colicins of *Escherichia coli* and nisins of lactic acid bacteria (Riley and Wertz 2002). It has been found that almost every bacterium derived from natural and clinical isolate is a colicin producer (Klaenhammer 1988; Riley and Gordon 1992). Genetic elements encoding toxins and corresponding immunity components are often located on bacterial plasmids. The mechanisms responsible for killing activity in bacteria and yeast are different. In bacteria, production of a toxin can cause lysis of the producer's cell, leading to large fitness costs incurred by the death of sizable subpopulations of producers. In contrast, killer yeast suffers relatively small fitness costs, typically no higher than ~3% (Wloch-Salamon et al. 2008).

Viral replication and toxin production incur metabolic costs impairing the ability of the host to compete for limited resources (Pintar and Starmer 2003; Wloch-Salamon et al. 2008). Thus, strains that do not benefit from carrying viruses are likely to be under selection pressure to lose them. That can occur when there is a low frequency of sensitive individuals in the environment, high number of toxin resistant cells, or when environmental resources are depleted. On the other hand, co-adaptation between the host and its virus might ameliorate these costs, especially if the transmission occurs only vertically, selecting for lower virulence. It can even lead to the co-dependence between symbionts causing so-called "addiction". This has been found for *Wolbachia* bacteria and an insect host (Pannebakker et al. 2007), and bacteria and their plasmid (Bouma and Lenski 1988). It has been shown that yeast that lost its killer viruses had altered gene-

expression patterns, indicating that coevolution between virus and yeast has led to changes in the host metabolism (McBride et al. 2013).

Spatial structure

The structure of environment has been found to be a crucial factor in the evolutionary success of killers (Chao and Levin 1981; Wloch-Salamon et al. 2008). In mixed liquid cultures, where dispersal is high, killers have an advantage only at high frequency (Adams et al. 1979; Czárán and Hoekstra 2003; Wloch-Salamon et al. 2008). Toxin producers competing at a low frequency are quickly eliminated from the population because concentrations of toxins are too low for efficient killing, while producers suffer from reduced fitness associated with toxin production. On agar surfaces, where dispersal is limited, toxin producers experience fitness benefits even at low frequency, because toxin accumulates at sufficiently high concentrations locally to kill surrounding non-producers (Chao and Levin 1981). Therefore, in such environments killers may successfully invade population of sensitives even being initially rare (Greig and Travisano 2008). The role of spatial structure in the interference competition has been also shown for other species, such as plants and insect (Amarasekare 2002).

Coevolution

Coevolution occurs when two or more species (or other units of biological organization, such as genetic elements or cells and their organelles) reciprocally affect each other's adaptive evolution (Thompson 1994). Studies on coevolutionary processes were initiated in the middle of the last century by investigating interactions between host plants and their pollinators (Ehrlich and Raven 1964; Janzen 1966). Coevolution is likely to develop when different species have close ecological interactions. An essential requirement for coevolution is the reciprocal nature of evolutionary changes in one species stimulated by the evolutionary change in the other species. Hence, coevolution refers to the mutual adjustments of separate genomes. Impressive outcomes of the coevolution between species include host plants and their pollinating *Heliconius* butterflies (Merrill et al. 2013), ants (Fischer et al. 2002), and wasps (Cook and Rasplus 2003). Coevolution happens at many levels, not just among separately living species. Intracellular interactions are particularly interesting examples, which include intracellular *Wolbachia* bacteria and their insect hosts (Pannebakker et al. 2007; Serbus et al. 2008), trematode parasites and their snail hosts (Koskella et al. 2011), bacteriophages and their *Pseudomonas fluorescens* (Buckling and Rainey 2002; Gómez and Buckling 2011; Hall et al. 2011) and *Escherichia coli* (Kashiwagi and Yomo 2011) bacterial hosts. Mitochondria, chloroplasts, and nuclei serve as other fascinating examples of intracellular symbiotic coevolution. Mitochondria are considered to have originated from proteobacteria and chloroplasts from cyanobacteria through endosymbiosis (Brindefalk

2009; Davidov and Jurkevitch 2009). Both organelles are closely connected in many ways to the intracellular metabolism of their hosts. Previously existing as independent units, they are now integrated with host metabolism to form a new tight symbiotic interaction (new unit). In case of mitochondria, coevolution between proteins encoded by the mitochondrial and nuclear genomes leads to the cell respiration, and changes in both organelles have been found to contribute to fitness of the host cell (Zeyl et al. 2005).

Coevolution can result in *mutualistic* relationships between species, in which both individuals benefit, or *antagonistic* (predator-prey, parasitism), in which one benefits at a fitness cost to the other. In antagonistic interactions, for instance as between predator and prey, selection usually goes towards an evolutionary arms race between prey and predator (Speed and Franks 2014). In host-parasite interactions, such as between nematode host and bacterial parasite (Schulte et al. 2010), as well as bacterial hosts and bacteriophages (Forde et al. 2008; Paterson et al. 2010; Marston et al. 2012), hosts can be particularly endangered as they have lower evolutionary potential (longer generation times) than their parasites (Gandon 2002). In such systems, an increase in fitness of one species causes a decline in fitness of species with which it interacts, which has been described as the Red Queen process (Van Valen 1973; Stenseth and Smith 1984; Woolhouse et al. 2002). This leads to greater phenotypic and genetic diversification in coevolving parasites and hosts, than can be observed in such populations evolving alone (Brockhurst and Koskella 2013).

Endosymbiotic relationships are thought to evolve towards mutualisms since they rely purely on vertical transmission and therefore fitness of endosymbionts fully depends on fitness of their host (Aanen and Bisseling 2014). Endosymbionts cannot afford antagonistic changes, because it would decrease their own fitness. The *Saccharomyces* killer system is an example of mutualistic relationship, in which coevolution between the host and its virus has led to a reduction of the costs associated with maintaining the virus. Similar decreases in fitness costs have been found in experimental evolution of plasmid-bacteria interaction (Bouma and Lenski 1988), and in *Wolbachia* and their insect hosts (Pannebakker et al. 2007). In sum, the examples mentioned above illustrate how changes in one partner select for the changes in the other, leading to coevolutionary responses. The dynamics of adjustments in these and other coevolving species depends on ecological conditions including the structure of environment in which the interaction takes place.

Coevolution of host-virus symbiosis in the "killer yeast" system

Endosymbiosis, involving the long-term stable and mutualistic interaction between a host and one or more inhabitant species (Nyholm and Graf 2012) is widespread in nature. Endosymbiotic relationships can vary in their complexity, involving one to hundreds or thousands of obligate or facultative enosymbionts living in the same host. Examples include legume roots and their nitrogen-fixing rhizobia (Gage et al, 2004), marine

sponges and their microbial symbionts (Webster and Taylor 2012), insects harbouring *Wolbachia* bacteria (Pannebakker et al. 2007), bacteria and their phages (Paterson et al. 2010) and plasmids (Bouma and Lenski 1988), and the microbial gut microbiomes of termites (Hongoh 2010, 2011) and humans (Marchesi 2010).

Endosymbiosis between killer viruses and their yeast hosts is thought to have developed from an initially more loose association. The yeast host can benefit from carrying the virus when competing with related yeasts that do not carry viruses (via produced toxins). The potential advantage results from removing competitors and releasing resources from their interior after they are killed (Ganter and Starmer 1992; Czárán and Hoekstra 2003; Wloch-Salamon et al. 2008). Viruses benefit from living in a cellular environment which allows them to survive and reproduce. Co-adaptation in such systems is at least partly driven by the selective costs and benefits for both partners of the interaction. However, the ecological importance of the killer yeasts and their close relatives is poorly known (Czárán and Hoekstra 2003). This makes it difficult to evaluate the benefits of toxin production (i.e. interference competition) relative to its fitness costs. Important but still unknown information necessary to understand the co-adaptation between yeast host and its virus, includes the killing efficiency of the killer yeast, the frequency of toxin-sensitive competitors in the environment (i.e. the prerequisite for toxin-related benefits), the evolvability of the "system", including possible trade-offs between interference and resource competitive ability, and whether evolution is more pronounced at the host-virus level or between killer and sensitive competitors.

Aims and outline

The general aim of the research presented in this thesis is to better understand the ecological and evolutionary significance of the killer yeast system. What are the costs and benefits of carrying the virus and producing the toxin, how are these costs and benefits affected by the coevolution between yeast hosts and their endosymbiotic viruses. I also address whether there are any constrains due to trade-offs between interference and resource competitive abilities, and which level of interaction in the killer yeast evolution is the most important: between yeast hosts and their viruses or between toxin-producing killers and non-producing sensitive strains. To address these questions, I have used strains of the genus *Saccharomyces*, derived from different habitats (Fig.4), in a number of laboratory experiments.

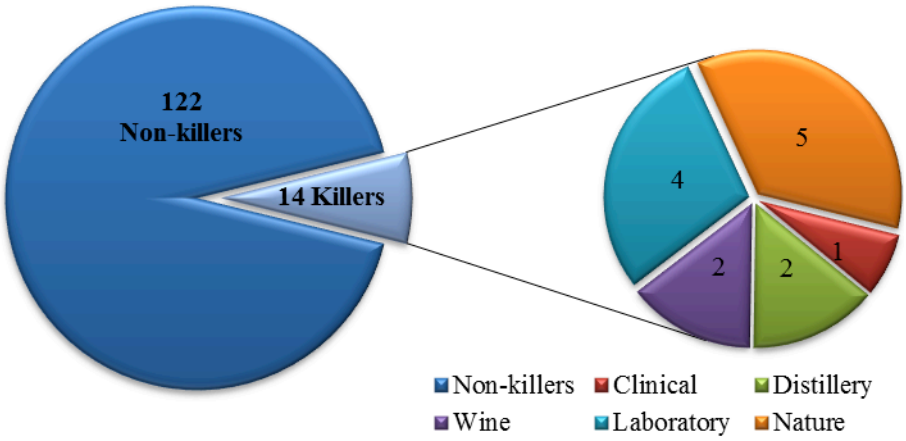

Figure 4. Number and sources of *S. cerevisiae* and *S. paradoxus* strains used in the thesis.

Chapter 2 describes the incidence and diversity of killer strains in two collections of yeast isolates from nature and man-made environments. I ask whether every virus affects every strain to the same degree or whether strains that carry cytoplasmic viruses differ from those that do not harbour viruses. This constitutes a test for co-adaptation between the host and its virus. I address this question by quantifying killing ability of isolates on both a sensitive laboratory strain and sensitive strains isolated from nature using the standard "Halo" assays. I also ask whether strains that do and do not carry host viruses differ in their susceptibility to standard laboratory yeast killer strains (producing K1, K2 and K28 toxin). I have found that in 10.3% of the yeast isolates viruses are found, and that toxin production and resistance to it do not appear to be genetically correlated.

Chapter 3 compares four assays devised to quantify the rate of toxic killing of a standard sensitive strain by killer strains of the three major types. I compare the classical "Halo method" (on agar) with three "Mixture methods" (in liquid culture) in terms of sensitivity and accuracy in discriminating between the three different killer types, as well as their reproducibility in independently replicated assays. I establish that "Halo method" continues to be especially attractive due to its easy application and low costs, and that it is also sensitive and reliable in quantifying the rate of toxic killing and in discriminating between toxin-producing strains.

Chapter 4 presents experimental tests of the co-adaptation between seven natural and one constructed yeast host strain and their "killer" viruses. The seven wild killer strains involve two *Saccharomyces* species: *S. cerevisiae* and *S. paradoxus* isolates, that all contain killer viruses producing the K1 toxin (derived fromisolates described in chapter 2). Killer viruses were isolated from their original hosts and used in cross-infections (after obtaining virus-cured versions of the hosts). The performance of native and foreign host-virus combinations were then tested by measuring virulence, competitive fitness, and the rate of virus loss in

a range of stresses. My tests present clear signatures of host-virus co-adaptation by exposing a visible reduction in virulence of newly created host-virus combinations, higher stability of evolved systems, and reduction in the competitive fitness of the newly established pairs. The last observation indicates that the initial metabolic cost of maintaining the virus has been turned into a partial dependence of natural host isolates on their killer viruses, indicating yeast hosts "addiction" to their virus partners.

Coevolutionary dynamics during a laboratory evolution experiment with a laboratory K1-killer and an isogenic non-producing strain of *S. cerevisiae* are described in **chapter 5**. I aimed at controlling the conditions shaping coevolution by manipulating the ability of either one or both strains to obtain and incorporate new mutations. Changes observed in the killing ability of the producer and in toxin sensitivity of the non-producing strain indicate that changes occurred in both of them providing that they were allowed to evolve. Coevolution resulted in an initial increase in the killing ability which was followed by a rapid increase in the frequency of toxin-resistant mutants which, in turn, led to suppressing the killing rate. Shifts in the competitive fitness of the evolved killer isolates showed a clear trade-off between the killing rate and the resource competitive ability. Moreover, by cross-infecting the killer virus between an ancestral and evolved strain, I was able to clearly demonstrate co-adaptation between a host and its killer virus.

Chapter 6 summarizes and discusses implications of the work presented in this thesis, including links between different studies and suggestions for future studies. I conclude that natural populations of *Saccharomyces* are likely confronted with widespread but not overwhelmingly prevalent killer-toxin producing competitors. As a result, local origin and maintenance of resistance are likely important in their ecology. The coevolution proved to be directly affected by reciprocal and adaptive responses of both partners. However, in order to fully understand the coevolutionary dynamics, comparative genomic studies of killer and resistant strains are needed. In particular, they could show whether the same genes are involved in natural and experimental evolution and whether required mutations tend to occur primarily within those host genes which are known to cause overexpression of killer phenotype and expression of resistance to the toxin. Another straightforward question is whether changes involve overexpression of viral particles or perfection of the protein toxin without altering viral numbers. Finally, an analysis of virus sequences could show whether there is any recombination between phylogenetically distinct viruses.

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Chapter 2

Incidence of symbiotic ds-RNA "killer" viruses in wild and domesticated yeast

Abstract

Viruses are found in almost all organisms and physical habitats. One interesting example is the yeast viral "killer system". The virus provides the host with a toxin directed against strains that do not carry it, while the yeast cell enables its propagation. Although yeast viruses are believed to be common, they have been actually described only for a limited number of yeast isolates. We surveyed 136 *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains of known origin and phylogenetic relatedness. Of these, 14 (~10%) were infected by killer viruses of one of the three types: K1, K2 or K28. As many as 34 strains (~25%) were not sensitive to at least one type of the killer toxin. In most cases, resistance did not disappear after attempts to cure the host strains from their viruses, suggesting that it was encoded in the host's genome. In terms of phylogeny, killer strains appear to be more related to each other than to non-killer ones. No such tendency is observed for the phenotype of toxin resistance. Our results suggest that even if the killer toxins are not always present, they do play significant role in yeast ecology and evolution.

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Virus elements that can be found in yeast cells include retroviruses, ss-RNA, and ds-RNA viruses. Most of them are non-infectious and apparently symptomless in their typical hosts; hence they are often named "virus-like-particles" (VLP's) (Ghabrial 1998). It has been repeatedly reported that their presence determines the production and secretion of low-molecular mass proteins and glycoprotein toxins (Makower and Bevan 1963). Toxins typically kill sensitive strains of the same and closely related species or genera (Schmitt and Breinig 2006). The so-called "killer phenotype" in *Saccharomyces* depends on the presence of ds-RNA viruses belonging to the *Totiviridae* family, a class of mycoviruses (Magliani et al. 1997). VLP's consist of two separately encapsulated ds-RNA viruses: an L-A helper virus and a toxin-coding M-satellite virus. The LA ds-RNA component of 4.6 kilobase-pair (kb) is an autonomously replicating virus and is responsible for encoding the capsid protein (Gag) and the viral RNA-dependent RNA polymerase (Pol). The M ds-RNA subunit of 1.5-1.9 kb is a satellite virus and contains genes for the production of toxins and associated immunity factors. The presence of both subunits together is required for the production of active toxin, which determines the killer phenotype of the host (Marquina et al. 2002). Mutants that have lost the ability to kill but at the same time harbour the resistance to killing, are named "neutral" (Schmitt and Radler 1990). They produce protein toxins, which are inactive due to defective mutations in the toxin gene of the M ds-RNA. VLP's tend to be lost at elevated temperature. In this way, normal killer stains can be "cured" of both toxicity and resistance while neutral strains of resistance only. The action of toxins is mediated by cell surface receptors (Schmitt and Radler 1990). The toxins kill sensitive yeast either by distorting the cell-membrane pH gradient or by blocking DNA synthesis and thus yeast growth. Based on killing-resistance profiles, three *Saccharomyces* viruses (ScV-M1, ScV-M2 and ScV-M28) have been characterized (Schmitt and Breinig 2002). Transfer of the virus is strictly vertical (Schmitt et al. 1996). Therefore killer viruses are inherited either after cell division or through mating with a donor cell, but not by "horizontal" infection (Wickner 1974, 1992).

Killer strains are thought to be ubiquitous in both *Saccharomyces sp.* and other yeast species. They have been incidentally found in cultures derived from the wild (fruits, mushrooms, spontaneous fermentation, soil, decaying plant material), as well as human-made habitats (Starmer et al. 1987; Schmitt and Breinig 2006; Vadkertiova and Slavikova 2007). We investigated two collections of *S. cerevisiae* and *S. paradoxus* strains, 136 in total, which were isolated from a variety of habitats including laboratories, soil, wineries, fermentation facilities, and human patients. The strains have been sequenced and therefore we knew how related they were in terms of phylogenetic distance (Liti et al. 2009; Schacherer et al. 2009). Our goal was to test which of the isolates showed a killer phenotype when confronted with a susceptible laboratory strain (i.e. one known not to harbor a killer virus). We also asked whether the strains that did and did not host viruses differed in their susceptibility to three known yeast killer strains (producing K1, K2 and K28 toxin). In this way, we could estimate how prevalent the viruses are and whether the phenotypes of toxicity and resistance are strictly associated with each other. Moreover, we hoped to see whether the

phenotypes of toxicity and resistance show a dependence on the ecological and phylogenetic differentiation of the host strains.

We used standard medium for the propagation of all strains, YPD broth, containing 10 g/L yeast extract, 20 g/L peptone, and 2 % glucose. YPD-MB agar (YPD containing 0.01% methylene blue and 1.5 % agar, adjusted with citric-phosphate buffer to pH=4.6) was used for assaying the killer activity and the presence/absence of resistance. This was done by seeding YPD-MB plates with cells of the sensitive M 984 strain and then overlaying a tested strain onto them. A zone of growth inhibition indicated toxin production and thus the presence of active virus. The next step was to classify the discovered killers into one of the three known phenotypes. This was done by introducing reference killers –K1, K2 or K28—hosted by the Y55 and MS300b strains. These were overlaid onto MBA plates seeded with a lawn of every discovered here killer strain. Resistance of a tested strain to a reference killer was confirmed if no signs of clearance thorough 3 days of incubation were seen. Our survey identified 14 strains infected with viruses (Supporting Table 1). Presence of the viral ds-RNA (Castillo et al. 2011), inferred from the observed toxicity, was verified by gel electrophoresis (Supporting Fig. 1). We also confirmed that the host strains can be cured of their killer phenotype by cultivation at increased temperature (37°C and 40°C) (Wickner 1974).

Figure 1 shows how the killer strains are distributed across different branches in the phylogeny. In the collection consisting of 71 strains (Liti et al. 2009), there were 5 killers. Of those, 1 was in *S. cerevisiae* and 4 in *S. paradoxus*. Among the *S. paradoxus* strains (Fig. 1b), the 4 killer strains appear to be generally closer to each other than to the remaining, non-killer, strains. To test whether this could be coincidental, we repeatedly drew at random 4 strains from an entire tree and calculated a mean phylogenetic distance between them. After 10,000 trials, we found that only in 4 random sets the distance was smaller than that actually observed. The type I error as low as $p = 0.0004$ suggests that the killers are indeed phylogenetically grouped. In another collection of *S. cerevisiae* strains, there were 5 killers located on a common tree (Fig. 1c). An analogous randomization test yielded $p = 0.0016$ and thus again indicated relatedness between the killer strains. In the latter case, however, the killer viruses were of three types: K1, K2, and K28. This precludes common single infection in the past. Rather, some related groups of strains are more likely to acquire, or maintain, viruses than others. Common environment is another potential factor. There were 28 strains isolated from wineries/bakeries (out of 60) and they contained as many as 4 killers (out of 5) (Fig. 1c). However, the sample of viruses is so small that is does not allow any conclusion about killers being more common in wineries/bakeries (Fisher's exact test, $p = 0.197$). No test is feasible for *S. paradoxus* because all strains of this species were isolated from virtually the same habitat.

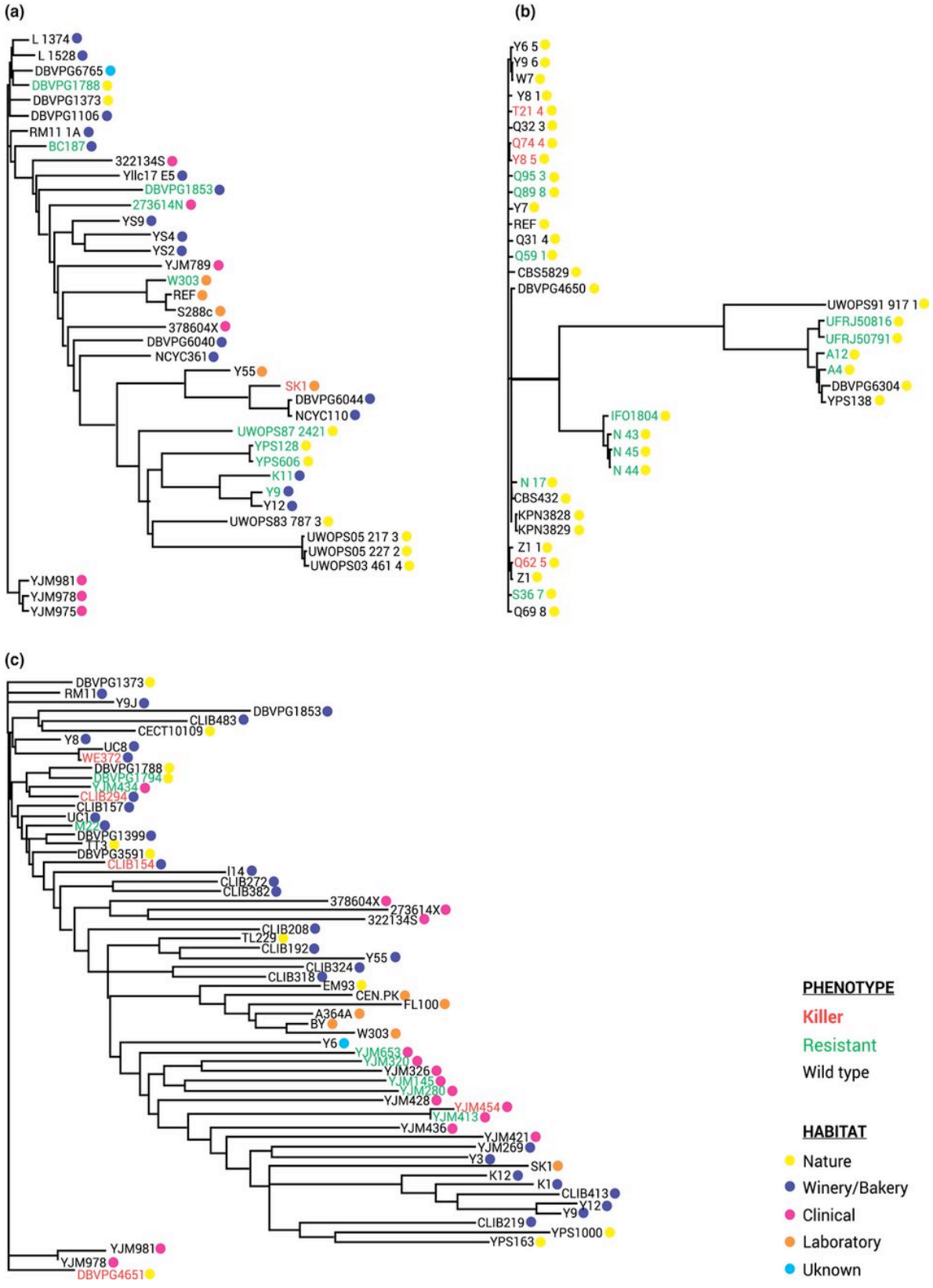

Figure 1. Phylogenetic relation between killer-producing and killer-resistant yeast isolates. Graphs a and b show, respectively, trees of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains from the collections of Liti *et al*. (2009). Graph c shows collection of *S. cerevisiae* strains of Schacherer *et al*. (2009).

Testing the 122 non-killer strains, we found that 88 of them were sensitive to all toxins, while the remaining 34 were resistant to at least one toxin (Supporting Table 2). Among the latter, 13 showed resistance to all killer types, eight to both K1 and K2, and eight to both K2 and K28. Of those resistant to only one toxin, a single strain was resistant to K28 toxin while 4 were resistant to the K2 toxin. The discovered phenotype of toxin resistance could have been coded by partly functional killer particles. To test this possibility, all the 34 identified resistant strains were subject to the standard protocol of virus curing through propagation at elevated temperature (37°C and 40°C). Only two of the assayed 34 strains lost their resistances and became sensitive to all three killer toxins. In contrast, all the 14 killer strains became sensitive to all three reference killer strains after applying the same procedure of curing. Considering how straightforward and repeatable curing of the 14 discovered killer strains (and the three reference strains) was, we suggest that the failure to cure the 32 resistant (and originally non-killer) strains indicates a chromosomal basis of this trait. It originated many times independently. This is suggested by randomization tests carried out in a similar, analogous to those described above. The observed distribution of resistance could result from chance with *p* equal to 0.635, 0.996, and 0.062 for the trees shown in Fig. 1a, 1b, and 1c, respectively.

To the best of our knowledge, the present survey of the yeast killer phenotype employed the largest number and the broadest diversity of *Saccharomyces* isolates. It revealed a rather low incidence (10.3 %) of the killer phenotype. Loss of killers after isolation is improbable. Any loss of VLP's alters the host gene expression and thus the stability of M ds-RNA (McBride et al. 2013). Indeed, the viruses appeared stable in our hands: they were difficult to cure with cycloheximidine and were never lost at the recurring events of freezing and thawing. We got rid of the viruses by applying severe heat stresses which was probably not experienced by any of these strains after their isolation. We think it is unlikely that the phenotype of being non-killer but toxin-resistant was determined by some overlooked by us viruses. Most of these strains were resistant to more than one toxin while virus-coded resistance is specific for the partner killer. This can be most likely caused by mutations in the host's genes, perhaps those associated with cell wall components (Page et al. 2003). In addition, our results reveal that resistance is not correlated in any obvious way with habitats from which they were isolated. Neither is genetic relatedness a factor, because the resistant strains lie on branches that were distant from the identified killers. In sum, we found that yeast killer viruses are relatively infrequent, while the resistance to them is rather common. This suggests that wild populations of *Saccharomyces* are confronted with the killer-toxin producing competitors at a rate sufficiently high to promote local origin and maintenance of resistance (Chao and Levin 1981; Czaran et al. 2002).

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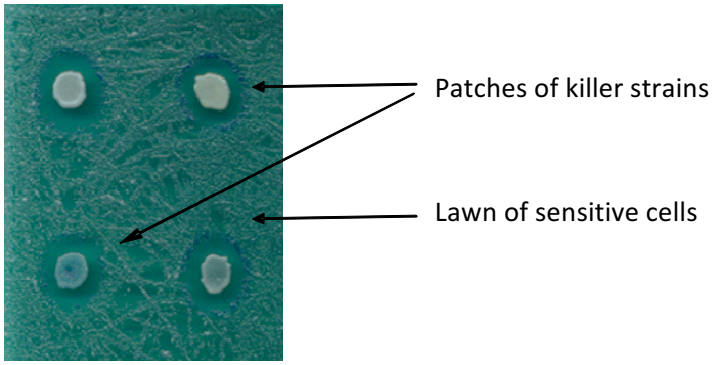
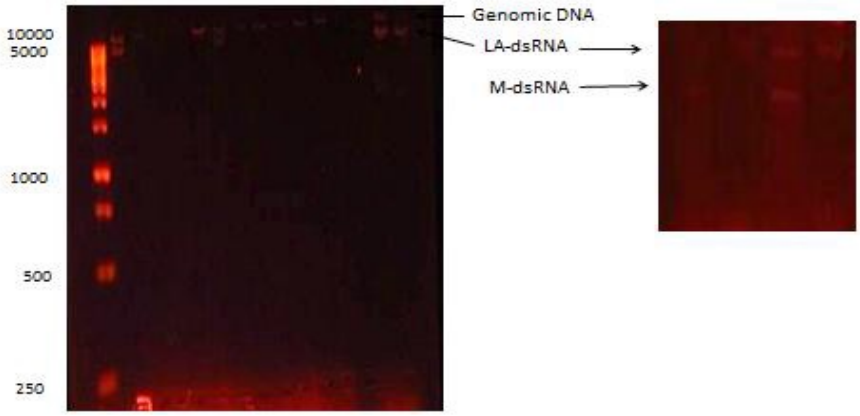
Supporting Figure 1

(a) Identification of the killer phenotype



(b) Detection of the viral dsRNA



Supporting Table 1. Killer strains

		Sensitivity to toxin producers			Inferred killer
	Source	**K1**	**K2**	**K28**	**phenotype**
S. cerevisiae					
Y55	Laboratory		+	+	K1
Y55	Laboratory			+	K2
MS300b	Laboratory				K28
SK1	Soil		+	+	K1
YJM454	Clinical		+	+	K1
DBVPG4651	Tuber sp.			+	K2
CECT10266				+	K2
CLIB294	Distillery		+	+	K1
CLIB154	Wine			+	K2
WE372	Wine			+	K2
S. paradoxus					
Q62.5	Oak woods		+	+	K1
Q74.4	Oak woods		+	+	K1
T21.4	Oak woods		+	+	K1
Y8.5	Oak woods		+	+	K1

Supporting Table 2. Toxin resistant strains

		Resistance to toxins			Curing-induced loss
Strain	**Source**	**K1**	**K2**	**K28**	**of resistance**
S. cerevisiae					
W303	Laboratory		+	+	
273614N	Clinical		+	+	
UWOPS87	Nature		+		
BC187	Fermentation	+	+		
DBVPG178	Nature/soil		+		
DBVPG185	Nature		+	+	
K11	Wine		+	+	
Y9	Wine		+		
YPS606	Nature		+	+	
YPS128	Soil		+	+	
YJM145	Clinical	+	+	+	+++
YJM280	Clinical	+	+		
YJM320	Clinical	+	+	+	
YLM413	Clinical	+	+		
YJM434	Clinical	+	+		
YJM653	Clinical	+	+	+	
YJM678		+	+		
CBS2888				+	
DBVPG1788	Soil		+		
M22	Wine	+	+		
PW5		+	+	+	
S. paradoxus					

Q59.1	Oak woods	+	+	+	
Q89.8	Oak woods	+	+	+	+++
Q95.3	Oak woods		+	+	
S36.7	Oak woods	+	+	+	
N-17	Oak woods	+	+	+	
N-43	Oak woods	+	+	+	
N-44	Oak woods	+	+	+	
N-45	Oak woods	+	+	+	
IFO1804	Oak woods	+	+		
A4	Oak woods	+	+		
A12	Oak woods	+	+	+	
UFRJ50791	Oak woods		+	+	
UFRJ50816	Oak woods		+	+	

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Chapter 3

Comparative analysis of methods to assess toxic killing ability in Saccharomyces killer strains

Abstract

Three dominant classes of cytoplasmic killer viruses have been characterised in *Saccharomyces*- K1, K2, K28 - each capable of forming a specific anti-competitor toxin and corresponding immunity factor.j To understand the ecological and evolutionary role of toxin production, its effect on competitors needs to be quantified, but methods that do so have never been adequately compared. We compare them in terms of sensitivity and accuracy in discriminating among the three different killer types, as well as their reproducibility in replicate assays. While each method quantifies the killing rate under different conditions, the classical "Halo method" performs best on both these accounts, while it is also the most convenient one being based on observations of growth on agar surfaces. The "Stationary-Phase Supernatant method" has the highest sensitivity and reproducibility of the three methods performed with liquid cultures. Three of the four methods indicate that K1 has the highest and K28 the lowest killing rate, which is consistent with previous accounts and shows that the proposed methods do not produce disparate results.

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Introduction

Saccharomyces cerevisiae carrying cytoplasmic ds-RNA "killer" viruses can kill related cells that do not carry viruses by the production and secretion of a low-molecular mass protein or glycoprotein toxin encoded by these viruses (Marquina et al. 2002; Santos et al. 2013; Wickner et al. 2013). Based on the killing mode of action, three principal *Saccharomyces* viruses (ScV-M1, ScV-M2 and ScV-M28), defined as killer viruses and belonging to the *Totiviridae* family (Schmitt and Breinig 2006; McBride et al. 2008; McBride et al. 2013), have been classified and described. Killing of a susceptible strain is a two-step receptor-mediated process. The first stage does not require energy input, and involves a quick binding of a heterodimeric protein to receptors that are present in the wall of sensitive cells. The main receptors for K1 and K2 toxins are β-1,6-D-glucan, while for K28 it is α-1,3-mannoprotein. The second, energy-dependent stage involves translocation of the toxin to the cytoplasmic membrane and interaction with its specific receptor. Binding of K1 and K2 toxins results in a series of physiological changes that lead to the death of sensitive cells. Initially, the disruption of the cytoplasmic membrane function, followed by leakage of potassium ions, release of ATP, reduction of metabolite levels contributes to the damage of the cell membrane pH gradient. Together these processes lead to the gradual death of sensitive cells. The mechanism of killing by K28 differs significantly from that of killing by K1 and K2. While K1 and K2 act on the surface of cells inducing ion-channels formation, K28 enters the cytosol by endocytosis. After binding to the receptor, it travels through the Golgi and endoplasmic reticulum to the cytosol where it sends a signal to the nucleus causing inhibition of DNA synthesis and cell cycle arrest. It blocks DNA replication during the cell-cycle which leads to the loss of cell viability (Schmitt and Breinig 2002, 2006).

To understand the ecological and evolutionary role of toxin production, it is essential to reliably assess the fitness costs and consequences for both the toxin producer and its toxin-sensitive competitors. Estimates of the killing rate of a toxin producer, i.e. the rate at which it kills sensitive strains, is especially important. Different methods have been used to estimate the killing rate of toxin-producing microbes. The "Halo method" estimates the size of the zone of no growth surrounding a patch of toxin producers growing on agar medium (de Ullivarri et al. 2011; Santos et al. 2011; Maqueda et al. 2012; Mehlomakulu et al. 2014). The "Serial-Dilution method" estimates the maximal dilution of droplets of spent liquid culture from a toxin producer that inhibits the growth of sensitive cells growing on agar (Schoustra et al. 2012). There are also methods for estimating the rate of killing in liquid cultures of sensitive cells confronted with toxin-producing strains or their products (Alfenore et al. 2003; Novotná et al. 2004; İzgü et al. 2006). For killer yeasts, the Halo method has been preferred to estimate the rates of killing (Kishida et al. 1996; Wloch-Salamon et al. 2008). This method is attractive due to its easy application and low cost, but is typically based on a single observation only. Its reliability across replicate experiments has never been systematically assessed and compared with other methods.

Here, we present work aimed at finding a method to estimate killing rates which would be convenient, sensitive and reproducible. Using the same set of killers and sensitive strains, media and conditions, we systematically compare four methods. Our results show that the classical "Halo method" has the best performance, yielding the highest "signal-to-noise ratio" of the four methods. This is somewhat surprising, since this method is based on a single observation after 72 h, whereas the other three methods measure the decline of sensitive cell numbers at multiple time points.

Materials and methods

1. Strains

K1 killer was hosted by a BY strain resistant to geneticin and nourseothricin (ho::kanMX4/ho::natMX4). An isogenic sensitive strain resistant to hygromycin B (BY, ho::hphMX4) served as a toxin-sensitive strain in subsequent assays (Wloch-Salamon et al. 2008). K2 was hosted by Y55 (MATa *ura1*); K28 was hosted by Ms300b (MAT α *leu2 ura3-52 ski2-2*) (Schmitt et al. 1996). We cured the K1 killer strain from its viral content (by growing cells at an elevated temperature), and used it as a non-killer control strain for all killers in the quantitative assays described below. All strains were obtained from the collection of the Institute of Environmental Sciences, Jagiellonian University.

2. Media

Liquid YPD was used to propagate cells prior to all toxicity assays. Liquid YPDG (YPD supplemented with 5% vol/vol glycerol to stabilise the toxin and adjusted with phosphate-citrate buffer to pH=4.6) was used in all assays of toxicity developed here. YPD agar supplemented with hygromycin B (ForMedium, UK) was used to count colony forming units of toxin-sensitive strains. MB (methylene blue) -YPD agar (pH 4.6) was used for the Halo assay.

3. Killing rate

In all four methods described below, killing rate of the three toxin-producing strains (K1, K2 and K28) was estimated using the same toxin-sensitive BY *ho*::hphMX4 strain. Experimental treatments and control treatments (the latter was not applicable for the Halo method) were replicated independently 10 times. To allow a meaningful comparison across the four methods, killing rates were expressed in terms of the number of killed sensitive cells per one killer cell per hour. We used linear regression to estimate the killing rate in assays employing liquid cultures. Other models –exponential, logarithmic, quadratic, and power—were also

tested but did not improve the fit to the data over a linear model, which explained 98%, 83% and 77% of the total variance for the K1, K2 and K28 killer strain, respectively ($P < 0.05$ in all cases).

3.1 Stationary-Phase Supernatant method

This method involved exposing stationary-phase sensitive cells to toxin-containing supernatant. To obtain the latter, a killer strain was grown in 1 ml YPDG for 24 h, the resulting culture was then added to 100 ml of fresh YPDG and grown for another 72 h. Cells were centrifuged at 3,500 × g for 10 min; supernatant was collected and filtered through a 0.45 µm sterile polyvinylidene fluoride membrane. The resulting cell-free supernatant with toxin was used directly in the test. A sensitive strain was grown for 48 h to stationary phase in liquid YPD, pH=4.6. Equal volumes of the stationary phase culture and toxin-containing supernatant were mixed and incubated at 25°C with gentle agitation. After 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 4 hours, adequate dilutions were made and plated on YPD agar. The number of resulting colonies was regressed against time without fixed intercept**.** The estimated slope minus the slope estimated analogously from untreated sensitive cell counts provided an estimate of the killing rate. To make it comparable with other methods, it was divided by the final number of killer cells (on average, 2 x 10^8 for every killer strain) used to obtain the volume of toxic supernatant applied in a single assay.

3.2 Logarithmic-Phase Mixture method

In this method, sensitive and killer strains were grown separately in liquid YPD, pH=4.6, until the logarithmic growth phase was reached. Equal volumes of both cultures were then mixed and further incubated with shaking. After 0, 0.25, 0.5, 0.75, and 1 hour, cultures were diluted and plated on media selecting for the sensitive or killer strain (after 1 hour buds started to separate from mother cells, obscuring the effect of killing). The killing rate of sensitive cells was estimated from the decline in sensitive cfu confronted with the toxin producer relative to the cfu of sensitive cells not exposed to the toxin producers. Because the latter kept replicating, the killing rate was s inferred by summing the rate of decline of the treated and the rate of increase of untreated cells (in both cases the sensitive ones). This summed rate was then divided by the number of killer cells present in the mixture at time point 0.75 h (i.e., on average 1.5 x 10^7 cells/ml).

3.3 Stationary-Phase Washed-Mixture method

Cultures of sensitive and killer strain were grown separately in liquid YPD to stationary phase (48 h). Cells were then washed with water to remove medium and toxins. The washed cells obtained from 0.5 ml of each of the two cultures were mixed, suspended in 1 ml of fresh YPD medium and incubated at 25°C with gentle shaking. After 0, 0.25, 0.5, 0.75, 1, and 1.5 h, aliquots were diluted and plated as described above. Killing was delayed in this assay, most probably because the cells had to enter log-phase to be fully sensitive to

toxins. We therefore estimated the killing rate from the slope connecting the last two time points only and dividing it by the number of killer cells applied (i.e., on average 2 x 10^8 cells/ml).

3.4 Halo method

For this method, sensitive and killer strains were pre-grown separately in liquid YPD, pH=4.6. Low-pH MB-YPD agar plates were then inoculated with 200 µl of a 100-fold dilution of stationary-phase sensitive cells (~4x10^5 cells). After the plates dried up, 5 µl aliquots of undiluted (~2x10^8 cells/ml) overnight killer culture were overlaid as small dots. The size of the halo produced around the killer patch was measured after 72 h of incubation at 25°C. Killing rate was inferred by estimating the surface area of the zone of growth inhibition (~8x10^3 sensitive cells per cm^2) at the time of inoculation divided by the number of killer cells (~1 x 10^6) and by the 72 h required for full formation of the halo.

Results

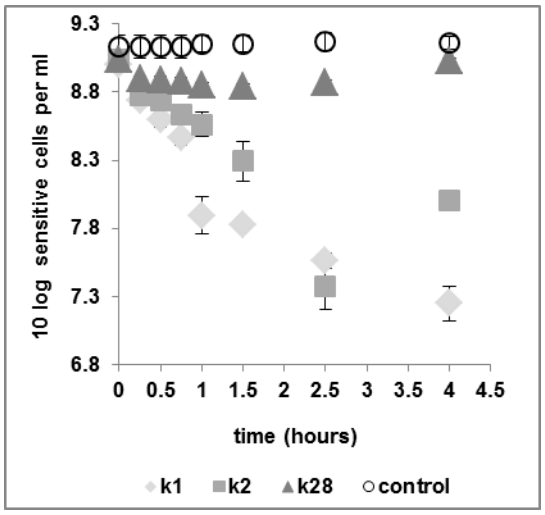
We estimated the killing rate of three yeast killer strains (K1, K2 and K28) on a single toxin-sensitive strain using four different methods: (i) Stationary-Phase Supernatant method, (ii) Logarithmic-Phase Mixture method, (iii) Stationary-Phase Washed-mixture method, and (iv) Halo method (see Methods for details). Fig. 1 presents results obtained for the four methods and three killer strains. Graphs show densities of sensitive cells for the first three liquid methods, and the average halo size for the fourth method arranged over 10 replicates. Clearly, the course of killing differs greatly for the three liquid methods: the Stationary-Phase Supernatant method and particularly the Logarithmic-Phase Mixture method show rapid declines in sensitive cell numbers, while the Stationary-Phase Washed-mixture method shows a delayed response. While the Halo method requires three days of incubation, compared with at most several hours for the other methods, it also requires the least workload, as it is based on a single observation (and therefore the dynamics of killing over 72 hours is not reported here).

We then estimated the killing rate for all three killer strains and four methods. This parameter quantifies the number of killed sensitive cells per killer cell per hour and can serve to compare the sensitivity and reproducibility of different methods. Fig. 2 presents averages and standard errors calculated for ten replicates. Clearly, the Halo method has the highest sensitivity, as producing the largest killing rates. The Stationary-Phase Supernatant method appears to be the best among the liquid-culture methods. All methods, except the Logarithmic-Phase Mixture method, indicate that K1 produces the highest and K28 the lowest killing rate. However, the ability to discriminate between specific killer strains differs substantially

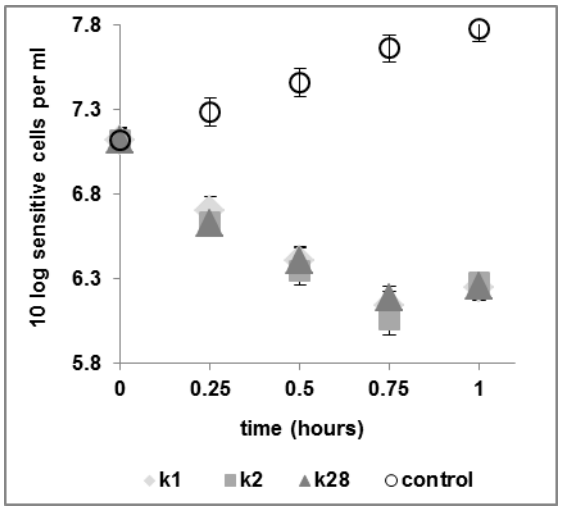
between methods. For example, the Stationary-Phase Washed-Mixture method finds a large difference between K1 and K2 or K28, but not between K2 and K28, while the Halo method finds a smaller difference in the killing rate between K1 and K2 and a larger difference between K2 and K28. To compare the sensitivity and reproducibility of the four methods, we calculated the coefficient of variation (CV) for each killer strain and method. Fig. 3 demonstrates that the Halo method has the smallest CV, hence the largest signal-to-noise ratio (i.e., combination of high sensitivity and high reproducibility). Among the other methods, the Stationary-Phase Supernatant method has the lowest while the Stationary-Phase Washed method has the highest CV.

To compare the statistical power of the four methods in discriminating between the killing rates of the different strains, we performed repeated-measures ANOVA. Table 1 shows the results of this analysis for the pairwise comparisons between the three killer strains and the single control strain. For all four methods, much of the explained variation refers to the differences between the control and killer strains, but all methods, except for the Stationary-Phase Washed-Mixture method, yielded also statistically significant differences between the killer strains. Overall, the power to discriminate between killer strains is highest for the Halo method, followed by the Stationary-Phase Supernatant method (which could not distinguish K28 from the control), whereas the Logarithmic-Phase Mixture method had the lowest statistical power.

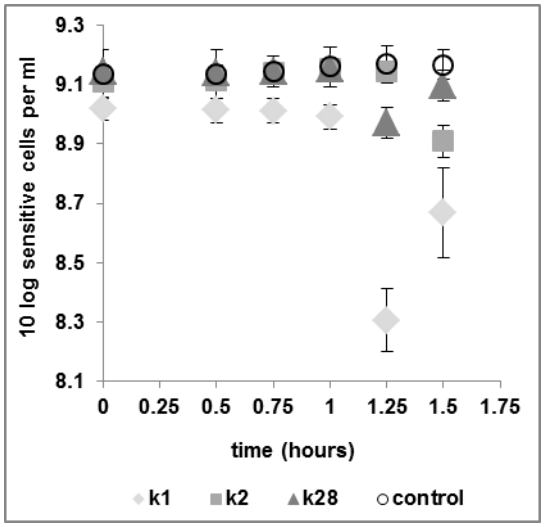
Stationary-Phase Supernatant method



Logarithmic-Phase Mixture method



Stationary-Phase Washed Mixture method



Halo method

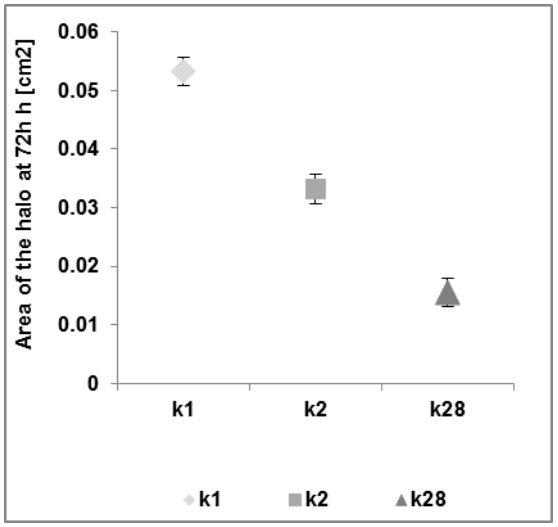


Figure 1. Survival of sensitive cells exposed to three killer strains (K1, K2 and K28). Error bars represent standard errors of means based on ten independent replications and are sometimes smaller than the symbol.

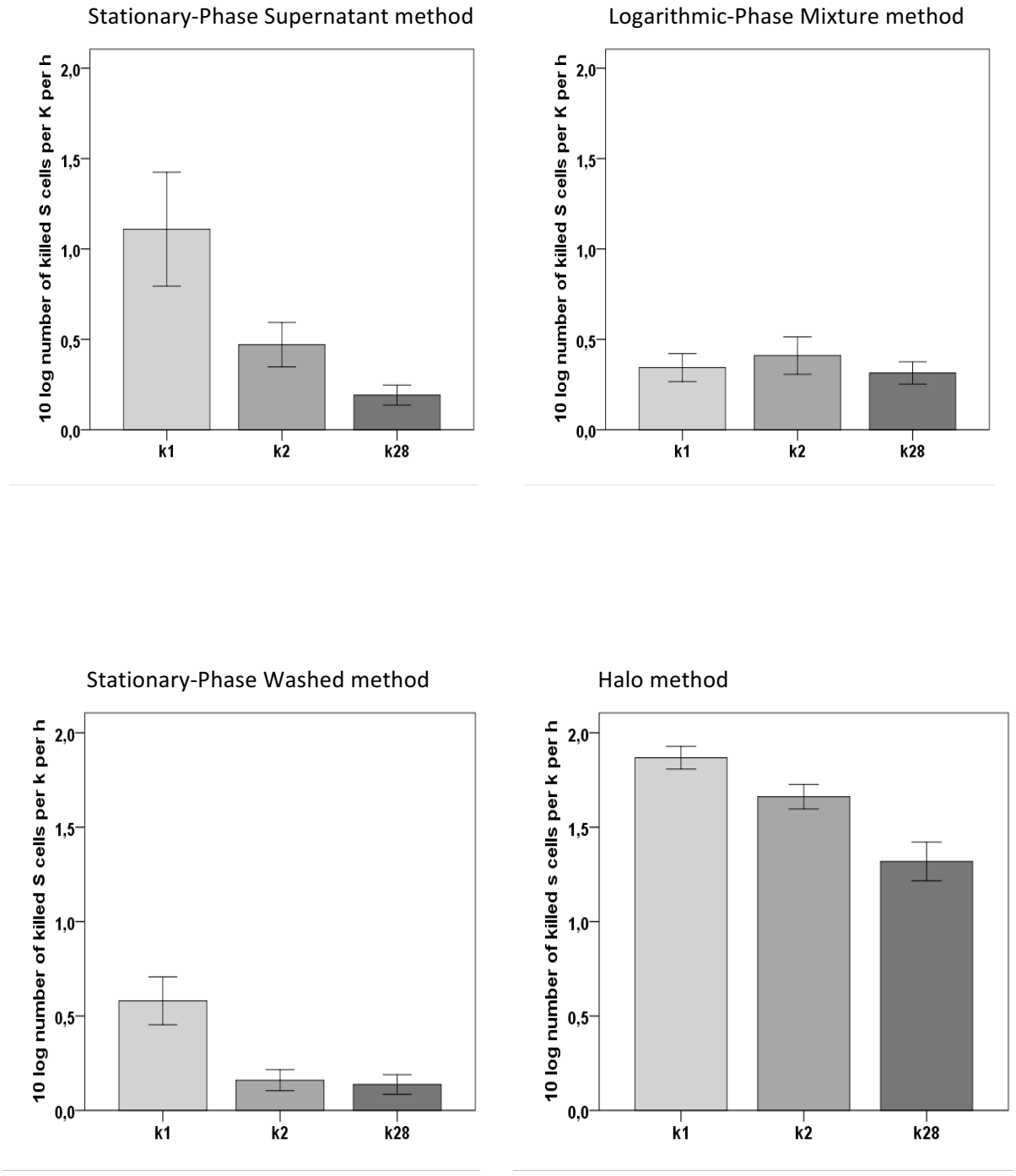


Figure 2. The "killing rate" estimates for killer strains K1, K2 and K28 obtained with four different methods (see Methods for details). Error bars represent standard errors of means based on ten independent replications.

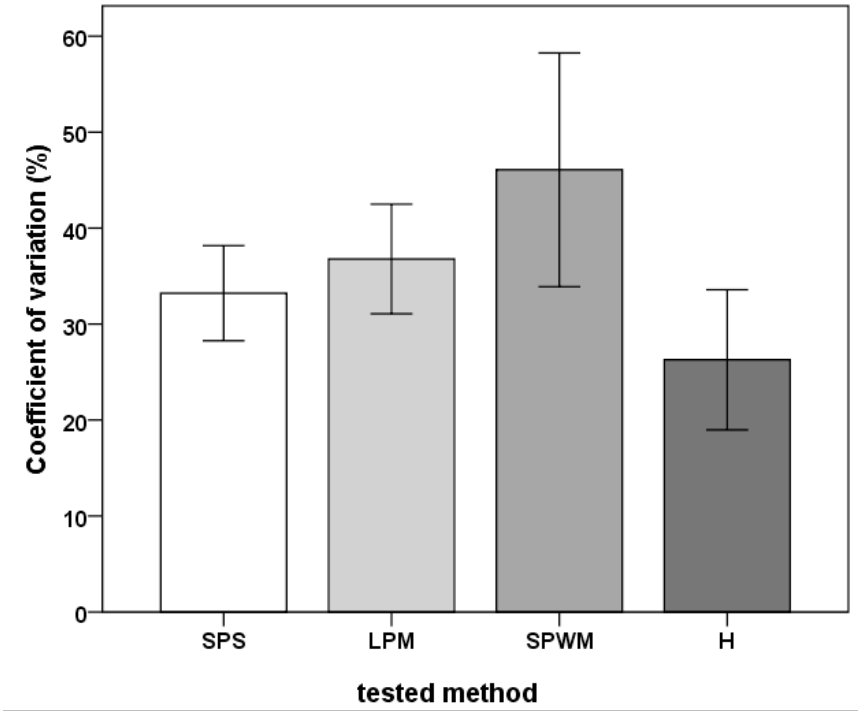


Figure 3. Coefficient of variation (CV) of 10 replicate estimates of the killing rate calculated for: SPS (Stationary Phase Supernatant), LPM (Logarithmic Phase Mixture), SPWM (Stationary Phase Washed Mixture), and H (Halo) methods. Low value of the CV is interpreted as a high signal-to-noise ratio and therefore relates to both sensitivity and reproducibility. Error bars represent standard errors of means based on the variation in CV among the three killers.

Table 1. Repeated-measures ANOVA. The table shows the overall fit of the model for each method and the *P*-values associated with all pairwise contrasts between control and killer strains.

Stationary-Phase Supernatant Mixture Method

F= 69.054	dfs=1,3		*P*<0.0001
	c	k1	*p*<.0.0001
		k2	*p*<.0.0001
		k28	*p*=0.383
	k1	c	*p*<0.0001
		k2	*p*<0.0001
		k28	*p*<0.0001
	k2	c	*p*<0.0001
		k1	*p*<0.0001
		k28	*p*<0.0001
	k28	c	*p*=0.383
		k1	*p*<0.0001
		k2	*p*<0.0001

Logarithmic-Phase Mixture Method

F=93.551	dfs=1,3		*P*<0.0001
	c	k1	*p*<0.0001
		k2	*p*<0.0001
		k28	*p*<0.0001
	k1	c	*p*<0.0001
		k2	*p*=1
		k28	*p*=1
	k2	c	*p*<0.0001
		k1	*p*=1
		k28	*p*=1
	k28	c	*p*<0.0001
		k1	*p*=1
		k2	*p*=1

Stationary-Phase Washed Mixture Method

F= 3.682	dfs=1,3		*P*=0.021
	c	k1	*P*=0.017
		k2	*p*=1
		k28	*p*=1
	k1	c	*p*=0.017
		k2	*p*=0.312
		k28	*p*=0.156
	k2	c	*p*=1
		k1	*p*=0.312
		k28	*p*=1
	k28	c	*p*=1
		k1	*p*=0.156
		k2	*p*=1

Halo Method

F=93.243	dfs=1,3		*P*<0.0001
	c	k1	*p*<0.0001
		k2	*p*<0.0001
		k28	*p*<0.0001
	k1	c	*p*<0.0001
		k2	*p*<0.0001
		k28	*p*<0.0001
	k2	c	*p*<0.0001
		k1	*p*<0.0001
		k28	*p*<0.0001
	k28	c	*p*<0.0001
		k1	*p*<0.0001
		k2	*p*<0.0001

Discussion

The production of anti-competitor toxins is a widespread phenomenon in microorganisms, yet we have limited understanding of its ecological and evolutionary role (Wloch-Salamon et al. 2008). Reliable estimates of the effect of toxin production are clearly needed; on toxin-sensitive organisms is an essential component for a better understanding of anti-competitor toxin production. To this end, we developed and compared four methods meant to quantify the rate of killing of toxin-sensitive cells. We find that the Halo method, which measures killing on agar and has been used most often in studies of killer yeasts, provides killing rate estimates that are most sensitive and reproducible (Fig. 3) and is best in discriminating the three killer strains (Table 1). Moreover, this method is especially convenient as it involves only a single measurement of the size of the zone of growth inhibition of the sensitive cells (i.e. the "halo"), whereas the other methods require measurements at multiple time points.

We can think of two possible reasons why the Halo method yielded estimates with the highest signal-to-noise ratio. First, on agar toxins can accumulate locally (i.e. close to the patch of killer cells) to much higher concentrations than anywhere in the three liquid-culture methods, where sensitive cells are mixed and each experiences the same and relatively low toxin concentration. Presumably, killing at low toxin concentrations is more stochastic per sensitive cell than killing at high concentration, causing clear and reproducible threshold zones of growth inhibition. Second, the course of growth of killer and sensitive cells

during the Halo assay may have contributed to more effective killing, because it was composed of both fast and slow phases of growth (Woods and Bevan 1968). Another possible explanation is that the toxin is relatively short lived and produced only in logarithmic phase. The relatively low killing rates estimated by other method using logarithmic-phase cells, the Logarithmic-Phase Mixture method, do not support this notion, although this may be also due to the generally lower toxin concentration in the liquid-culture assay.

The fact that the Halo method produces the most sensitive and reliable killing rate estimates does not necessarily imply that these estimates are most accurate - even under the specific conditions of the assay. One cause of inaccuracy could be that killing rates are based on a single observation after 72 h required observing a clear halo. Therefore, actual killing rates may be underestimated when killing stops or decelerates gradually before reaching this time point. Another complicating factor is the growth and budding of killer and sensitive cells during the assay. We expressed the killing rate using the original numbers of sensitive and killer cells. This assumption is likely incorrect, since it will take time for the toxin to be produced and diffuse from the patch of toxin-producing cells, while the number of both cell types likely increases. However, measuring the actual local dynamics of toxin production, diffusion and killing would require far more advanced and not yet tried methods. We observed that the size of the halo does not change much from the time when it just becomes visible (~48 h) and when we measure it (72 h). This suggests that the initial period of incubation is most critical. It also means that great care must be taken when optimizing densities of cells and volumes of aliquots at the moment of inoculating the killer and sensitive cells on agar surfaces.

Despite differences in cell physiology and spatial structure of the killer and sensitive cells between methods, three of four methods consistently found the highest killing rate for the K1 and the lowest for K28 killers. The fact that the Logarithmic-Phase Mixture method does not yield differences in killing rate among the three killer strains is interesting. It indicates that the physiological state of the cell also determines the toxin concentration required for killing. The Halo method (also involving growth conditions, but higher local toxin concentration) and the Stationary-Phase Supernatant method (also involving low toxin concentration, but different cell physiology) do reveal differences in killing rate. It is possible that postponed production or longer half-life of the K1 toxin, relatively to K28, means that it takes more time to develop toxicity of K1 even if it is generally stronger. The relatively high effectiveness of K28 under growth conditions may also partly result from its different molecular mechanism: whereas toxins K1 and K2 are known to act by forming ion channels in the plasma membrane (Flegelová et al. 2002; Santos and Marquina 2004), toxin K28 enters the cytosol by endocytosis, blocking DNA synthesis and growth, which contributes to the loss of cell viability (Eisfeld et al. 2000; Schmitt and Breinig 2002). It is likely that under growth conditions the endocytotic uptake of K28 and its further action are more effective than in the stationary phase. Finally, it should be admitted that we applied only one specific set of environmental conditions for all killers. Under different

conditions, relative strengths of toxins could be different. However, this should not bias our results considerably. Of all environmental parameters, pH is especially important for the action of toxins. We applied pH=4.6, because pH of 4-5 has been reported as optimal irrespective of the type of killer (Bussey et al. 1979; Tipper and Bostian 1984; Golubev and Shabalin 1994; Marquina et al. 2002; McBride et al. 2013). It was also demonstrated that the competitive gain of toxin production was lost at a pH outside this range (Greig and Travisano 2008; McBride et al. 2008; McBride et al. 2013). Moreover, pH=4.6 proved effective for scoring the killer phenotype when large collections of wild yeast strains, some containing killer viruses, were assayed (Pieczynska et al. 2013).

While our experiments highlight the value of the classical Halo method, the estimates produced by it should be used carefully when interpreting the fitness of toxin production in different environments, because the dynamics of competition between killer and sensitive strains may differ quantitatively under different conditions. Much more advanced methods would be required to measure the dependence of killing rates on specific environmental and physiological conditions. With this cautionary remark, the Halo method is a sensitive, reliable and convenient method for at least the initial detection, and discrimination between, the killer strains of yeast.

Acknowledgments

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Chapter 4

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

Abstract

Many fungi carry cytoplasmic viruses that encode anti-competitor toxins. These killer viruses provide certain benefits to their host, but also incur metabolic costs associated with viral replication, toxin production and immunity. What causes the stable maintenance of these endosymbionts is insufficiently understood. Here, we test whether adaptation between host and killer viruses causes their stable maintenance in seven natural and one laboratory strain of the genus *Saccharomyces*. We use transfection of killer viruses, all encoding the K1-type toxin, among these isolates to test three predictions from co-adaptation. Our results show clear signs of host adaptation to their killer viruses in all three tests. First, we find strong reductions in virulence against a standard sensitive strain for new relative to native host-virus combinations, with higher virulence for transfections within than between yeast species. Second, we observe a lower probability to loose viruses under stress for native than for new host-virus combinations. Third, and perhaps most remarkably, we find positive effects on competitive fitness from introducing native viruses after curing, but negative effects when foreign viruses are introduced. These results indicate that natural killer strains have adapted and even become “addicted” to their killer viruses, which may explain their stable association.

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Introduction

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

The yeast killer system is an interesting example of a mutualistic symbiosis. In this system, yeast cells host cytoplasmic M virus like particles (VLPs), which encode anticompetitor toxins (producing K1, K2 or K28 toxin and corresponding immunity component), and LA helper virus, which is responsible for encoding the capsid proteins and the viral RNA-dependent RNA polymerase (Schmitt and Breinig 2002, 2006; McBride et al. 2013). The yeast host can benefit from toxin production when competing with other yeasts that do not carry killer viruses, possibly in two ways: by removing competitors for the primary limiting resource, as well as by liberating resources from killed competitors (Wloch-Salamon et al. 2008). Conversely, viruses depend for their fitness entirely on their host, since they can no longer escape their host and infect new hosts (Wickner 1996), except during outcrossing, which is thought to be infrequent in yeast (Zeyl and Otto 2007). At the same time, virus carriage initially incurs a fitness cost, which is probably associated with the metabolic costs involving viral replication, toxin production and immunity (Wloch-Salamon et al. 2008). Therefore, in the absence of toxic killing, selection may break the association and cause the loss of the killer virus. However, since virus fitness depends strongly on host fitness, host fitness costs of carrying the virus are expected to diminish over time (McBride et al. 2013).

Coevolution between host and killer virus can stabilize the association, for instance when compensatory evolution removes the cost of virus carriage or even causes “addiction” (i.e. fitness reduction in the host after removal of the virus) if the benefits of compensatory mutations are specific and conditional on the presence of the virus. This was observed during evolution of a bacterium-plasmid association, where the plasmid initially incurred a cost upon the host, but co-adaptation caused the bacteria to become “addicted” to the plasmid, such that plasmid removal incurred a cost (Bouma and Lenski 1988). Analogously, compensatory evolution has been frequently observed within the same genome in antibiotic-resistant bacteria (Andersson and Hughes 2010) and toxin-resistant fungi (Schoustra et al. 2007), where initial fitness costs associated with toxin resistance are removed during laboratory evolution, sometimes leading to decreased fitness after removal of the resistance mutation (Schoustra et al. 2007). Another example of

coevolution leading to "addiction" was shown for the association between Wolbachia bacteria and their insect hosts, where the host became infertile after removal of the endosymbiont (Pannebakker et al. 2007).

Two factors determining the potential for coevolution are the evolutionary time spent together as symbionts and the genomic target size involved in their interaction. Moreover, ecological conditions where the association provides host benefits, such as spatially-structured environments and sufficient densities to allow frequent encounters with sensitive competitors in case of yeast-killer association (Greig and Travisano 2008; Wloch-Salamon et al. 2008), will facilitate the initial stage of co-adaptation when the mutual dependence of symbionts is still low. Yeast killer strains were traditionally considered ubiquitous and present in nearly every environment tested: fruits, mushrooms, spontaneous fermentation, soil, decaying plant material, industrial and laboratory collections (Schmitt and Breinig 2002). We have recently tested this expectation by examining more than one hundred isolates of *S. cerevisiae* and *S. paradoxus* from laboratory collections, nature, vineyards, clinics and industry. We found that while only a minority of yeast strains carried killer viruses (10.3%), there were relatively many strains which were resistant to viruses (25%), suggesting that killer viruses are a significant factor in yeast evolution (Pieczynska et al. 2013). With respect to genomic target size, a number of yeast nuclear genes, belonging to the MAK, KEX, SKI families are required for the efficient maintenance, replication and expression of the killer phenotype (Wickner 1992; McBride et al. 2013), but many more genes may affect the host-virus association through effects on general metabolism.

Here, we test for signatures of coevolution between yeast host and their toxin-encoding viruses in the yeast killer system. We use cross-infection of viruses among eight killer strains, seven wild isolates (Liti et al. 2009; Schacherer et al. 2009) and one constructed killer strain (Wloch-Salamon et al. 2008), to study effects on host virulence (killing phenotype) and fitness and the stability of the host-virus association. Our results show clear signatures of host adaptation to their native virus in all tests performed: lower killing rates, higher viral loss rates during stress and lower competitive fitness for new relative to native host-virus combinations. Most strikingly, whereas introduction of foreign viruses decreases fitness, introduction of native viruses increases fitness in all natural isolates, but not in the newly constructed killer strain, indicating host "addiction" to their native virus. Finally, we find a positive correlation between competitive fitness and virulence among these strains, suggesting that their long-term association has removed possible fitness costs of virulence.

Materials and Methods

Strains

Table 1 lists all strains used. These include a previously constructed K1 killer and isogenic (except for selectable markers) toxin-sensitive strain of *Saccharomyces cerevisiae* (Wloch-Salamon et al. 2008) and seven wild strains of *S. cerevisiae* and *S. paradoxus* which carried killer viruses and were derived from two yeast strain collections that have been sequenced (Liti et al. 2009; Schacherer et al. 2009). These killer strains were found in natural habitats, a distillery and clinic and all harboured viruses encoding K1-type toxin (Pieczynska et al. 2013). The laboratory K1 strain served as a control lacking an evolutionary history together with its viral symbiont. The sensitive laboratory strains served as a reference strain for measuring killing rate (i.e. sensitivity to the toxin, described below) and as a control for the effect of curing strains from their viruses. The fully resistant to the toxin laboratory reference strain (Pagé et al. 2003; Wloch-Salamon et al. 2008) was used in fitness competition assays to measure fitness of native and newly infected killer strains. All killer strains were "cured" from their viral contents before the cross-infection experiment, which was done by propagating each strain for three days at an elevated temperature.

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 co-adaptation experiment. Low-pH YPD solidified with 2% agar was used for the competition experiments to estimate fitness and for the stability assay using single-cell transfers. To estimate competitor numbers in the fitness assay, SC (synthetic complete) medium with 1% 5-FOA was used to score colonies with uracil auxotrophy, and SC medium without uracil to score for colonies with uracil prototrophy. SC without uracil was also used to select for transformants in the transfection experiments.
Assays of killing rate, so called "Halo" assays, were done with low-pH YPD supplied with 0.003% MB (methylene blue) and solidified with 2% agar.

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 (1M sorbitol, 0.1M EDTA, 0.1 M Na2SO4, 0.8 M KCL, pH=7.5) and suspended in 10ml of the PKE buffer (30mM Na2HPO4 150 mM KCL, 10 mM EDTA, pH 7.6). Cells were treated with 0.1%-1% non-ionic detergent (Np40) and incubated for 1h at 30°C. Disrupted cells were centrifuged at 4,000 g for 30 min at 4°C to separate supernatant from the rest of the 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-infection of killer viruses

The *pAG60* plasmid with the selectable *URA3* gene (Goldstein et al. 1999) was used to check for successful cross-infections. Laboratory killer and sensitive strains were already uracil auxotrophs, however all wild killers were prototrophs. Therefore, in order to inactivate the uracil synthesis pathway, all wild killers were transformed with amplified *URA3* cassette from the laboratory sensitive strain, following lithium acetate procedure (Gietz et al. 1995). To begin cross-infections, cells were collected from exponentially growing cultures by low-speed centrifugation (3000 g) and washed four times with water. Cells were then suspended in 1M LiAc and immediately collected by centrifugation at 13,000 g for 30 sec. 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 of 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 8,000 g, suspended in YPD and immediately spread on SC-uracil plates. After 3 days of incubation at 30°C, colonies were picked, followed by immediate assessment of killing rate and clones showing killer phenotype, thus carrying killer viruses were store at - 80°C for propagation further assays.

Assay of killing rate

Low-pH MB-YPD agar plates were inoculated with 200 µl of a 100-fold dilution of YPD stationary-phase culture of sensitive cells (~$4x10^5$ cells per plate). After the plates dried up, 5 µl aliquots of undiluted (~$2x10^8$ cells/ml) overnight killer cultures were overlaid as small patches. The size of the halo produced around the killer patch was measured in millimetres after 72 h of incubation at 25°C. Virulence was expressed as killing rate by the number of killed sensitive cells (estimated from the area of the zone of growth inhibition assuming the initial density of ~$8x10^3$ sensitive cells per cm^2) divided by the number of killer cells initially present (~1 x 10^6) and by 72 h, that is, the time period during which the halo developed. The assumption that only the initially present killer and sensitive cells are involved in the halo development is likely incorrect, but the standardization of conditions and use of the same sensitive strain across assays make these estimates a valuable measure of relative killing rate.

Co-adaptation experiment

To equilibrate newly constructed host-virus combinations after transfection, we allowed each new strain to adapt physiologically under standard growth conditions that are optimal for the production and activity of the K1 toxin (YPD with pH 4.6, 25 °C). Each killer strain was cultured under these conditions and 1% (~$2x10^6$ cells) was transferred to fresh medium every 24 hours for ~ 50 generations in total (eight transfers).

Assay of competitive fitness

Relative fitness was measured by pairwise competitions between tested strains and the standard toxin-resistance reference strain with different antibiotic-resistance marker (Pagé et al. 2003; Wloch-Salamon et al. 2008). Both strains were grown separately by transferring 1% of a stationary phase culture (~$2x\ 10^6$ cells) to YPD agar plates (pH=4.6) and incubating for 24 h at 25 °C, in order to adjust strains to the conditions of the competition environment. Cells were then washed off agar plates with 10 ml of water, mixed in equal proportion and 10 µl (~$2x10^6$ cells) was spread on fresh low-pH YPD agar plates, which were incubated for 48 h at 25 °C. The frequency of both competitors was 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 the resistant strain (laboratory reference 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

Three conditions were applied to the original and newly transfected killer strains that are known to increase the loss of killer viruses. First, growth at three increased temperatures (38°C, 40°C, 42°C) was used (Wickner 1974). For this, strains were grown on YPD agar plates for three days, after which single colonies (ten for each strain) were screen for the loss of killer phenotype, thus loss of killer viruses by standard Halo method (Kishida et al. 1996). Second, three concentrations of cycloheximide (0.3 µg/ml, 0.5 µg/ml, 1 µg/ml) were applied (Fink and Styles 1972). Similar to the assay using increased temperature, also here strains were grown on YPD agar plates supplemented with an appropriate concentration of cycloheximide, for three consecutive days, after which same assessment of the loss of killing phenotype was applied on single clones from each tested strain. Finally, killer strains were subjected to a series of 10 single-cell transfers to minimize effects of selection between host cells carrying varying titres of viruses. This was done on YPD agar with three replicate lines per strain by streaking single colonies every 72 h on fresh medium (allowing ~20 generations during colony growth between transfers). Viral loss was determined for all strains and stress

conditions using the halo test using a standard sensitive strain, where the complete absence of a halo was scored as a loss of viruses.

Statistical analyses

We used 2-sample *t-tests* for testing the effect of strain (native and new hosts) on the killing rate and competitive fitness. To test the effect of fitness from curing from viruses, we employed paired *t-tests*. Two-way ANOVA analysis with transfected killer viruses and host as fixed factors was used to compare their relative effect on virulence and fitness. Pearson's correlation was used to test the relationship between the killing rate and fitness. Fisher's exact test was conducted to assess the effect of stressors on the loss of killer viruses for native and cross-infected strains.

Results

We performed transfection of toxin-encoding killer viruses to test for signs of co-adaptation between yeast and its native virus, using seven natural virus-carrying strains from various sources as donor and the same seven strains together with a recently constructed killer strain and its isogenic sensitive version as recipient (Table 1). These strains were picked from sequenced collections of *S. cerevisiae* and *S. paradoxus* strains (Liti et al. 2009; Schacherer et al. 2009), which were recently tested for killer phenotype and found to be of the most common K1 type (Pieczynska et al. 2013). The strains were "cured" from their killer viruses, viruses were isolated and used in an attempt to construct all 63 (i.e. seven donors and nine recipients) possible donor-recipient combinations. Of the transfections, only 36 were successful due to problems either with viral isolation or transfection (Table 1): we were unable to isolate viruses from one of the wild strain (SK1), despite its successful transfection with other wild viruses, and three of the donors (Q62.5, Q74.4, and CLIB294) could not be infected with any of the viruses, including their own, despite positive control transformations with a plasmid.

Table 1. Cross-infected strains used in the experiment. Scores of plus indicate successful transfection with own/foreign viruses.

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

*** All strains were originally planned to be used as both donors and acceptors but some of them failed in one of these roles, see Results.**

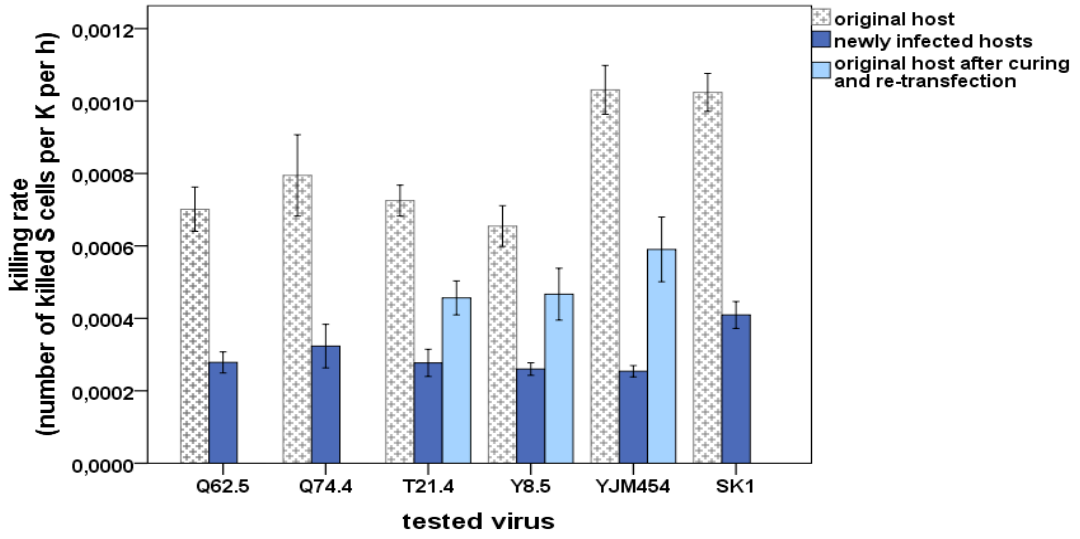
Killing rate

With this collection of native and transfected K1 killer strains, we performed three tests. We first compared killing rate of the 36 new with the six original host-virus combinations against a standard sensitive laboratory strain using a halo test (Figure 1 and Supplementary Figure 1). In all cases, the native combination had a higher killing rate than the new combinations (Fig. 1A; 1-sample *t-tests* comparing the virulence for various foreign viruses against the native virus for each host: 2-tailed $P<0.01$ for all six strains). However, killing rate was also lower for the successful re-infections of the native viruses (Figure 1A), and we subjected all combinations to 50 generations of growth to allow for physiological adaptation (e.g. equilibration of virus titre). Figure 1B shows that the three successfully re-created host-virus combinations (T21.4, Y8.5, and YJM54) substantially increased their killing rate during this period, reaching similar virulence as the original strains (paired *t-test*: $t=-5.94$, df=2, 2-tailed $P=0.027$). The re-created original combinations were more virulent than the newly created combinations after adaptation (*t-test* comparing the mean virulence of native and foreign combinations for the three viruses that successfully re-infected their native host: $t=-4.17$, df=2, 2-tailed $P=0.014$). Interestingly, the sensitive reference strain S shows lower killing rate after transfection than the constructed killer strain K1, even though they are isogenic except for an antibiotic

marker (Supplementary Figure 1; t=5.15, df=10, 2-tailed P<0.001), suggesting that K1 adapted to its viruses during its short history together as a laboratory strain.

Since killer strains came from two species of *Saccharomyces* (S. *paradoxus* or *S. cerevisiae*), we tested whether killing rate reduction from the introduction of foreign viruses was smaller among transfections between donor and recipient from the same relative to different species. Figure 2 shows that indeed the 13 transfections within the same species caused lower reductions in killing rate than the 20 transfections between these two species (t=2.45, df=31, 2-tailed P=0.020), suggesting a common genetic component in the adaptation of yeast hosts to their viruses.

A



B

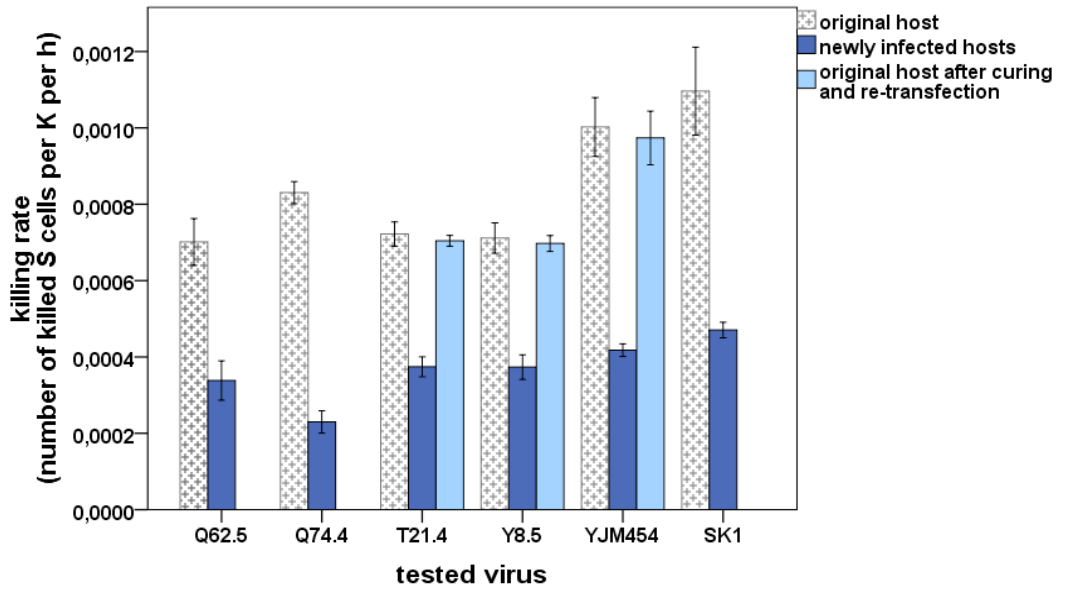


Figure 1. Killing rate of original and new host-virus combinations measured against a reference toxin-sensitive strain. (A) Killing rate estimates of original and new host-virus combinations immediately after transfection into new hosts, (B) killing rate estimates after allowing ~ 50 generations of physiological adaptation following transfection into new hosts; original strains were also subjected to additional adaptation. Shown are mean and standard errors of the mean based on three independent measurements per strain.

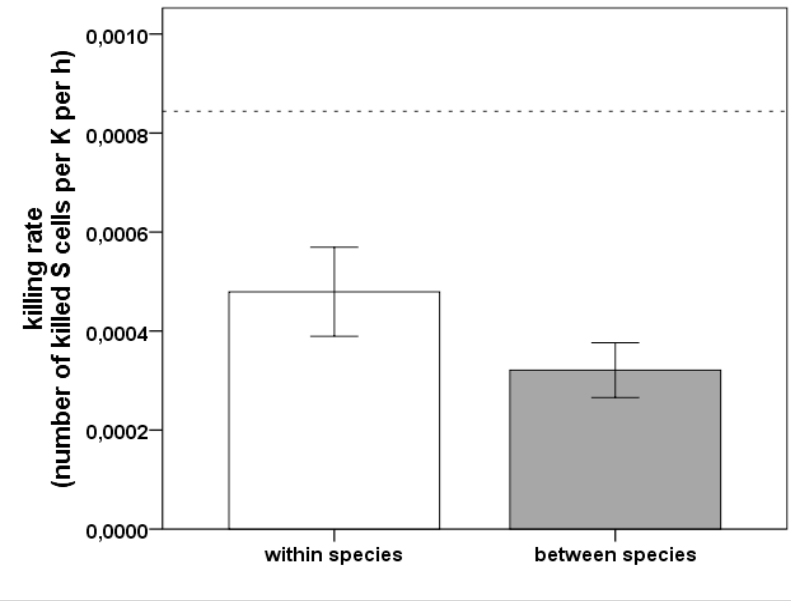


Figure 2. Killing rate of new host-virus combinations created by transfecting viruses between donor and recipient strains of the same or different yeast species (*S. saccharomyces* or *S. paradoxus*). Killing rate was measured against a standard toxin-sensitive strain with three-fold replication for 13 within-species and 20 between-species transfections (see Table 2). Error bars represent standard errors of the mean virulence of the 13 and 20 transfections involved. A dashed reference line shows the average killing rate of the eight native combinations.

Host fitness

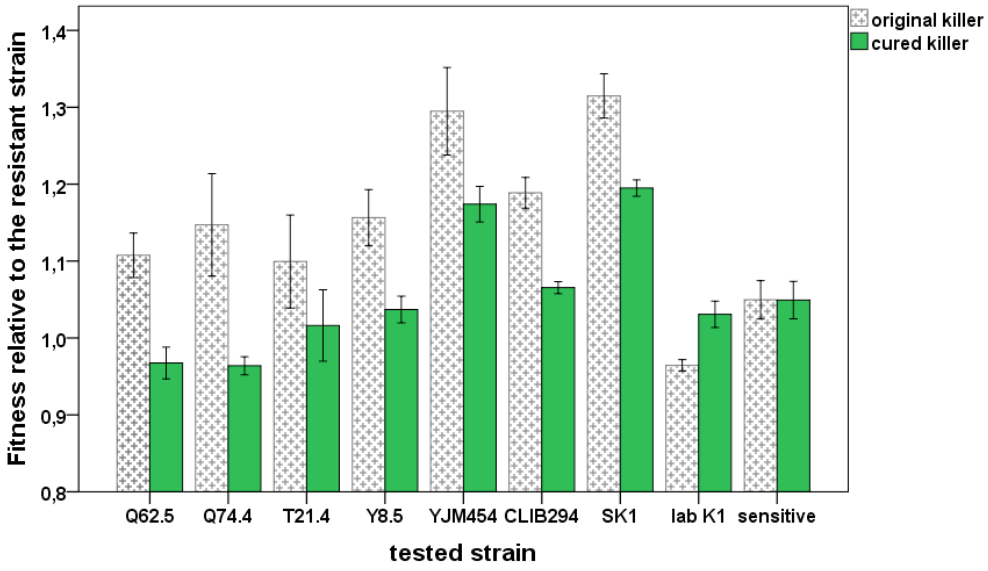
Next, we tested the effect of native and foreign viruses on host competitive fitness. For this, we performed competition experiments between (native or constructed) killer strains and a reference toxin-resistant strain on standard YPD agar medium in the absence of sensitive cells (i.e. in the absence of killing benefits); competitions were done on agar instead of liquid culture to compare results with a previous study for constructed killer strain K1 (Wloch-Salamon et al. 2008). Figure 3A shows the relative fitness of the eight killer strains and the one sensitive strain (as control), before and after curing the strains from their viruses. The curing procedure itself did not affect fitness, which can be seen from the lack of curing effect on the fitness of the sensitive control strain (t=0.19, df=4, 2-tailed P=0.986). Consistent with previous results (Wloch-Salamon et al. 2008), the constructed K1 laboratory strain increases in fitness when cured (t=-7.09, df=4, 2-tailed P=0.0021) to a level that is indistinguishable from that of the sensitive strain (t=1.24, df=4, 2-tailed P=0.282), whereas in contrast the natural killer strains show a decrease in fitness (paired t-test: t=6.93, df=6, 2-tailed P<0.001). Moreover, the six strains that were successfully transfected with viruses from *S. paradoxus* strains T21.4 and Q74.4 showed further declines in fitness upon receiving the new virus (Figure 3B; paired *t-tests* comparing mean fitness without and with foreign virus: for Q74.4 t=6.27, df=5, 2-tailed P=0.0015; for T21.4 t=9.02, df=4, 2-tailed P<0.001). When the relative contributions of host strain and virus

on fitness and virulence were compared in transfections with these two viruses, both host and virus had a significant effect, but host variation had a much stronger influence (Table 2).

Table 2. Two-way ANOVA testing the relative effect of yeast host and virus (for the two successfully transfected viruses Q74.4 and T21.4) on relative fitness and killing rate.

Tested effect	*F*	dfs	*P*
Killing rate			
Host	18.355	5,5	<0.001
Virus	4.697	1,5	0.040
Host*Virus	4.255	1,5	0.007
Relative Fitness			
Host	131.614	5,5	<0.001
Virus	4.428	1,5	0.046
Host*Virus	2.372	1,5	0.070

A



B

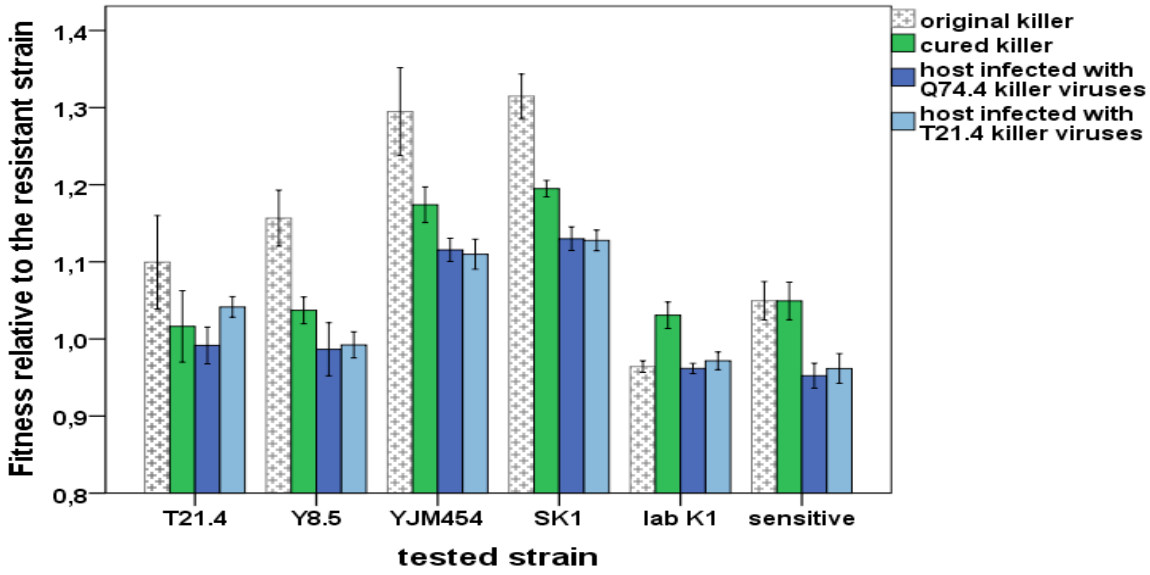


Figure 3. Effect of viral carriage on fitness of the host measured in competition against a reference toxin-resistant strain in the absence of toxic killing on standard YPD agar medium. (A) Effect of curing the eight original virus-carrying hosts from their viruses; a toxin-sensitive strain without virus was used as control for the method used for curing. (B) Effect on fitness of introducing two new viruses (from *S. paradoxus* donor strains Q74.4 and T21.4) to six strains for which transfection was successful; fitness was measured immediately after transfection. Error bars represent standard errors of the mean based on three independent assays.

Since viral carriage initially incurs a fitness cost (see laboratory strain K1 in Figure 3A), we were interested whether virulence strength may have similar costs. We examined this for the seven killer strains with their native viruses and the constructed K1 killer strain by testing for a correlation between killing rate and fitness. Figure 4 shows that, in contrast, there was a significant positive correlation between killing rate and fitness for these presumably co-adapted host-virus associations (Pearson's r=0.818, n=8, P=0.013), showing that whatever fitness cost of virulence there may have been initially, these have been removed by later adaptation.

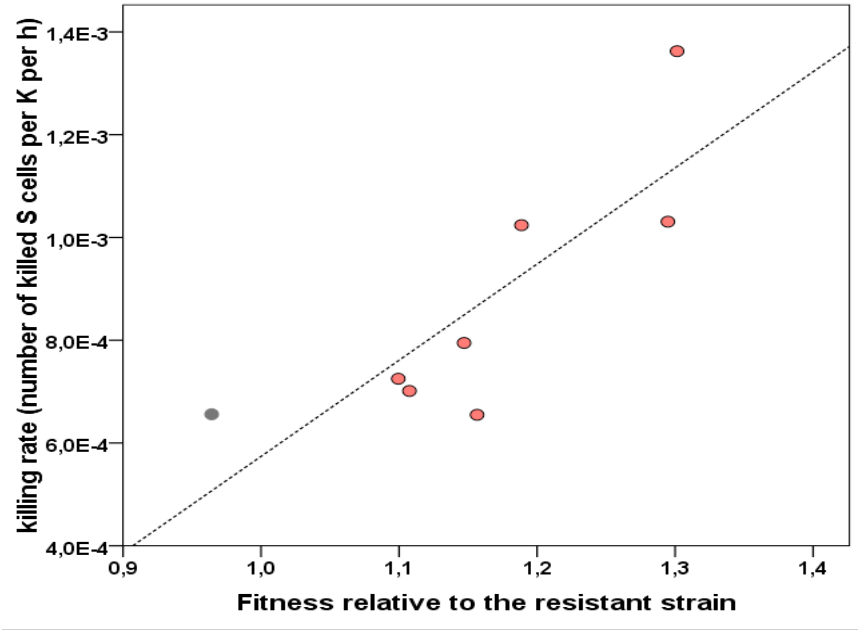


Figure 4. Relationship between killing rate and competitive fitness for the eight original host-virus combinations. Fitness is measured in direct competition experiments against a toxin-resistant reference strain under standard laboratory conditions in the absence of toxin-sensitive cells. The positive correlation is significant (r=0.818 , n=8, P=0.013).

Stability of host-virus associations

Finally, we reasoned that co-adaptation of host and viruses may have increased the stability of their association. We tested this by comparing viral loss rates for native and new host-virus combinations under three conditions known to increase the rate of virus loss: elevated temperature (38°C, 40°C, 42°C), three concentrations of cycloheximide (0.3, 0.5 and 1 µg/ml), and 10 serial-transfers of colonies through single-cell bottlenecks (to minimize the effect of selection among host cells, including for hosts that have lost their killer viruses). Viral loss was scored using halo tests showing the complete absence of a halo. Supplementary Table 1 lists viral losses across conditions and strains. We noted that at 38°C and a cycloheximide

concentration of 0.3 µg/ml differences in viral loss were most pronounced, and used these to score viral loss. Fig. 5 shows that the original host-virus combinations were significantly more stable under elevated temperature, cycloheximide application and single-cell transfers than the 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 eight original versus 36 new combinations). The clearest difference in stability was observed after the single-cell transfers. Here, the viruses were lost in the majority of the new combinations, while in all original and reconstructed original combinations the viruses persisted.

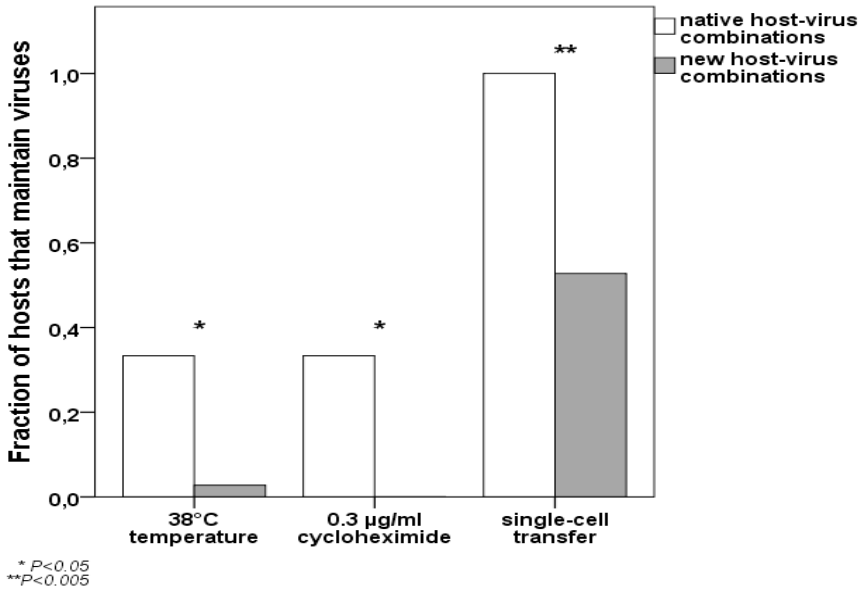


Figure 5. Stability of host-virus association in original and new hosts using three different stress conditions (growth at high temperature or in the presence of cycloheximide and 10 single-cell transfers). Shown is the fraction of host-virus combinations where viruses were maintained for the eight original and 36 new combinations. Viral maintenance was determined in a classical halo test against the standard sensitive strain by scoring viral loss by the complete absence of halo.

Discussion

We conducted a cross-infection study with seven wild and one laboratory yeast killer strains belonging to two species, *Saccharomyces cerevisiae* and *S. paradoxus*. All eight strains harbour the M virus-like particle encoding K1 toxin together with LA helper virus in their cytoplasm and are able to kill cells from a standard sensitive reference strain under certain conditions. By exchanging killer viruses among these eight strains, we were able to make 33 killer strains harbouring new host-virus combinations (not all transfections were successful even after several attempts). Virulence assays showed that virulence is higher for the original

host-virus combinations than for the new combinations. Measurements of their competitive fitness in the absence of toxin killing indicated also clear negative effects of introducing new relative to native viruses. Moreover, removal of the viruses showed a positive effect in the laboratory killer strain, where host and virus did not share an evolutionary history and this effect likely reflected the metabolic costs of viral carriage. In contrast, removal of the viruses from wild strains had a negative effect on fitness, while introducing foreign viruses had even stronger negative effects than curing strains from their native viruses, showing that wild strains were "addicted" to their killer viruses. Finally, viral loss rates were much higher for new than for native host-virus combinations under conditions that stimulate loss. These results consistently indicate co-adaptation between the wild strains and their killer viruses.

A complicating factor for comparing performance of native and new host-virus combinations was that new host-virus combinations seemed to need some time to "equilibrate" or adapt in order to show optimal performance. This problem appeared when hosts transfected with their own native viruses showed lower killing rates immediately after transfection than after 50 generations of growth in benign conditions, when they reached killing rates similar to the original strains (Figure 1 and Supplementary Figure 1). Lower performance immediately after transfection may be due to the effective transformation of few viral particles (only a fraction of the viral supernatant was used for each transfection). This may be particularly problematic, when the original virus population was genetically or epigenetically diverse and this diversity is required for full host performance (Vignuzzi et al. 2005).

The negative fitness effect from removing the killer viruses from the wild strains is remarkable and exemplifies that host and virus have become obligate mutualistic symbionts: the virus is not able to escape its host and infect other hosts (McBride, 2013), while the fitness of the host – even in the absence of toxic killing – decreases without the presence of the virus. Dependence between symbionts has evolved in many other systems, such as during the transition from facultative parasitism to obligate mutualism between Wolbachia and a parasitic wasp (Pannebakker et al. 2007). Also, bacteria are dependent on their plasmids if they carry genes for toxins with a longer half-life than that of the antidote they also encode (Van Melderen and De Bast 2009), or when compensatory mutations for the metabolic cost of plasmid carriage occur in the bacterial genome that are specific and deleterious in the absence of the plasmid (Bouma and Lenski 1988). McBride et al. (2013) showed that the loss of co-infection of yeast by L-A and M viruses led to alterations in host gene-expression pathways, indicating that coevolution between virus and yeast has led to changes in host metabolism.

Interactions between coevolving symbionts are often antagonistic when the fitness of each partner does not (fully) depend on the other (Van Valen 1973; Stenseth and Smith 1984). However, once such dependence has been established, such as for an endosymbiont being unable to spread to other hosts except via vertical transmission to the offspring of its host, natural selection is expected to prevent further

antagonism from the side of the dependent symbiont (Maynard Smith and Szathmáry 1995). This is also a likely scenario for both partners in the yeast killer system. The killer virus has become almost entirely dependent on its host, since horizontal transmission (to new hosts) has become very infrequent: no extracellular route of infection is known (Wickner 1996) and outcrossing happens at very low frequency (Zeyl and Otto 2007). The dependence of the host on its killer virus is less strong, but still significant: the killer virus enlarges the habitat of yeast by allowing toxic killing of resource competitors, while removal of the virus incurs a fitness cost even in the absence of toxic killing (see Figure 3).

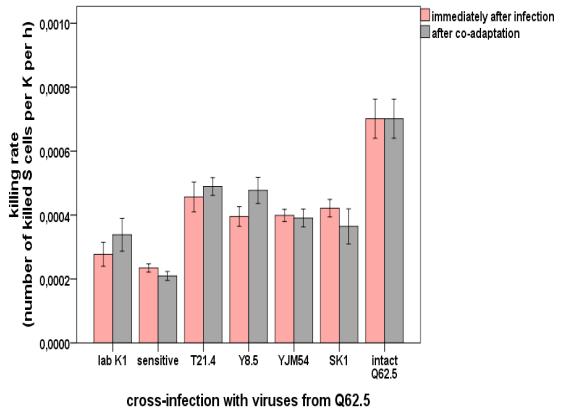
Although it seems likely that adaptive changes occurred in both symbionts during their shared evolutionary history, without temporal information we cannot be certain (Janzen 1980). Indirect support that both symbionts changed evolutionarily comes from the fact that both different hosts and different killer viruses caused changes in virulence and fitness (Table 2). However, we cannot rule out that this variation existed before the symbiosis, or that the genetic changes occurred during the symbiosis but not in response to each other. A decisive test of the coevolution between host and killer virus would entail controlled evolution experiments, where changes in both symbionts relative to their ancestral state could be monitored, and their causes be verified in competition experiments where fitness effects of mutations could be tested in the absence and presence of the other symbiont (e.g. (Paterson et al. 2010). We are presently performing such experimental study, which should further enlighten our understanding of the dynamics of coevolution in the yeast killer system.

Acknowledgments

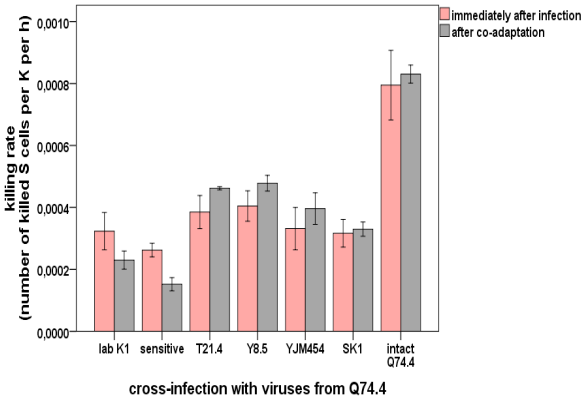
We thank Richard Kormelink for helpful comments and Jan van Lent for technical assistance. This work was supported by the Foundation for Polish Science, “International PhD Projects”, grant no. MPD/2009-3/5, and the Graduate School for Production Ecology and Resource Conservation (PE&RC), Wageningen University.

Supporting Figure

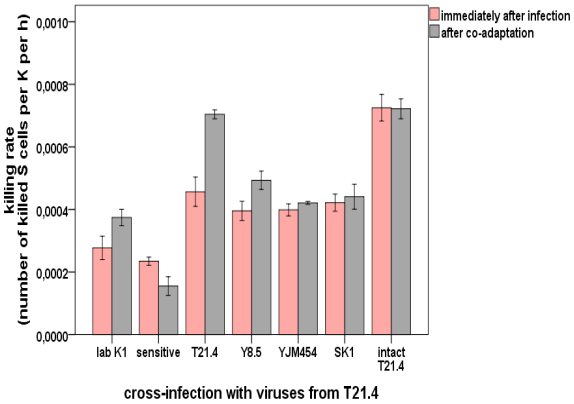
a)Q62.5



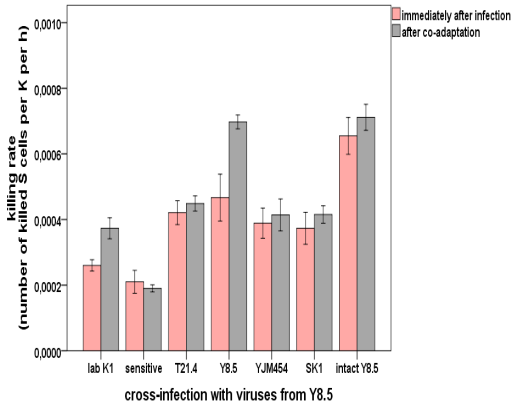
b)Q74.4



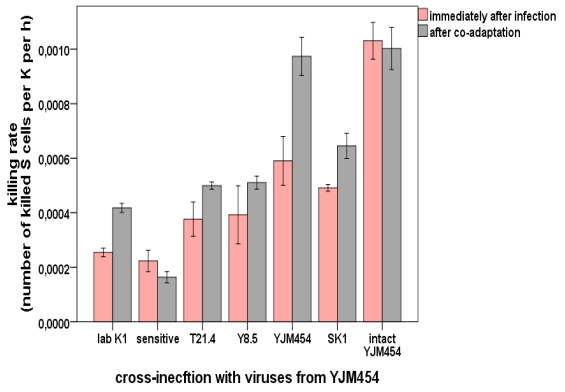
c)T21. 4



d)Y8.5



e)YJM454



f)CLIB294

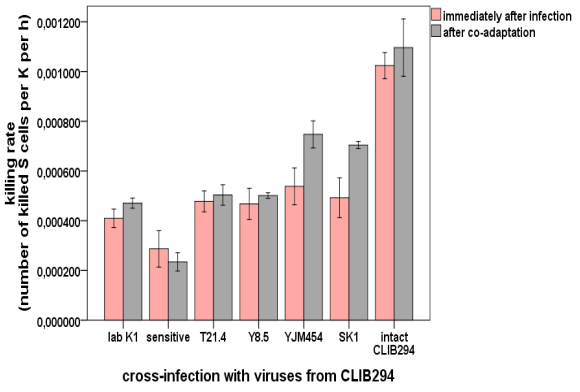


Figure 1. Comparative analysis of killing rates in the co-adaptation experiment between newly infected and original killers. Variations estimated for each of 5 cured killers and sensitive strain after cross-infection with one of 6 wild viruses: (A)Q62.5, (B)Q74.4, (C)T21.4, (D)Y8.5, (E)YJM454, (F)CLIB294, respectively, in the comparison with the original killer. An assay performed twice: immediately upon completing cross-infection study (left graphs), and after subjecting all combinations to physical co-adaptation experiment (right graphs). Error bars represent standard errors of the mean based on ten independent replications

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Chapter 5

Rapid multiple-level coevolutionary dynamics in experimental populations of yeast killer and non-killer strains

Abstract

Coevolution between different biological entities is considered an important evolutionary mechanism at all levels of biological organization. However, empirical demonstrations of reciprocally evoked evolutionary changes in multiple partners are scarce and involve mostly comparative analyses. Here we report changes evolved in a yeast killer strain (K), which carries cytoplasmic dsRNA viruses coding for anti-competitor toxins, and an isogenic toxin-sensitive strain (S) during 500 generations of experimental evolution. By allowing only one or both strains to evolve, we manipulate the opportunity for coevolution to occur and test for its signatures at two levels: between K and S strains and between host and virus in the K strain. Changes in killing rate of K, toxin sensitivity of S and resource competitive ability indicate coevolution at both levels. First, only in populations where both K and S are allowed to evolve, killing rates rapidly increase accompanied by the rapid invasion of toxin-resistant mutants, which subsequently drive a strong decline in killing rate. Sporulation of resistant and sensitive cells shows that resistance evolved via the substitution of two subsequent mutations. Fitness measurements show that increases in killing rate are associated with metabolic costs in the absence of sensitive cells (forcing the divergence between resource and interference competitive strategies), but provide selective benefits in the presence of sensitive cells. Second, swapping the killer virus between the ancestor and an evolved strain with high killing rate shows changes in both host and virus that are positive only when combined, indicating reciprocal coevolution of host and virus. Together, our results demonstrate the potential for rapid and simultaneous coevolutionary dynamics at multiple levels in yeast killer strains.

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Introduction

Coevolution is the process of reciprocal adaptation between species or other biological entities. It may vary in complexity, involving either two or more species affecting each other's evolution (Thompson 1994). Understanding the factors that stimulate the rate and dynamics of coevolution allows to maintain species in multiple ecological settings. Coevolutionary processes may occur at diverse levels of biological organisation, 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 microbes (Hongoh 2010; Marchesi 2010), hosts and their parasites (Decaestecker et al. 2007; Schulte et al. 2010; Koskella et al. 2011) cells and their organelles such as mitochondria (Zeyl et al. 2005), and bacteria and their bacteriophages and 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 (mutalism). 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), while the ability of parasites to escape from their present host and infect new hosts is an important determinant of their virulence since it affects the association between virulence and fitness l(Aanen and Bisseling 2014).

Viruses, which are 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 replication and survival. Viruses may coevolve with their hosts in various ways, one possible result being a stable endosymbiotic relationship (Ghabrial 1998; Pearson et al. 2009). However, often viruses can escape their host and spread to new hosts, such as 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 lead to an accelerated pace of genome evolution, especially in the genes that encode virulence and host-protection (Barrick and Lenski 2013). Furthermore, previous studies on bacteria-phage interactions show that phages evolve faster, thereby sometimes increasing the mutation rate of their bacterial host when bacterial host and phage are allowed to coevolve (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 killer 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 responsible for toxin production and antidote to it, the second encoding capsid proteins and RNA-dependant RNA polymerase), which together determine the killer phenotype (Magliani et al. 1997; Marquina et al. 2002). The anti-competitor toxin is effective against sensitive (non-killer) strains that lack virus elements. Competition via anti-competitor 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 a crucial role in the maintenance of microbial diversity (Adams et al. 1979; Czárán et al. 2002; Kerr et al. 2002; Pintar and Starmer 2003). Non-producing toxin-sensitive strains loose in competition against killers, because they are eliminated by the produced toxin. However, since viral replication and toxin production involve metabolic costs, non-producers have a higher resource competitive ability in the absence of killers (Pintar and Starmer 2003).

The outcome of competition between toxin-producers and non-producers depends on resource availability and the frequency of their encounters, which in turn depends on the spatial structure of the environment, since yeasts are not motile. When dispersal is low, toxin producers benefit from the effect of killing more than non-producers, since they are closest 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 in a positive frequency-dependent way 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 different levels: driven by interactions between toxin-producer "killer" strain and non-producer "sensitive" 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, where both strains compete indirectly for limiting resources, but only the killer strain is able to interfere directly with non-producers 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 previous study on killer yeast illustrates that loss of viruses by yeast hosts results in changes in the yeast genome, indicating the integration of virus and host metabolism due to coadaptation between host and virus (McBride et al. 2013). Additionally, our recent study on cross-infection of killer viruses among natural isolates of killer strains demonstrates that after sufficient evolutionary time the fitness costs of carrying killer virus become compensated and may even lead to "addiction", i.e. increased fitness when the virus is present (chapter 4 of this thesis). However, the mechanism and causes of coevolutionary dynamics in the yeast killer system remain poorly known.

Here, we use experimental evolution to study the role and dynamics of coevolution in the yeast killer system. We are particularly interested in the evolvability of both interference and resource competitive

ability under conditions where killer and sensitive strains interact frequently. We allow populations of a constructed *Saccharomyces* killer and isogenic non-killer strain to evolve during 500 generations in a structured environment, while we manipulate the opportunity for coevolution. We allow either both strains or only one strain to evolve in mixed populations (cf.(Rice 1996), and use populations of only killer or sensitive strain as further controls. By weekly resetting the ratio of killer and sensitive strain in the mixed populations to 1:10, we maximize opportunities for interaction by preventing the disappearance of the sensitive strain. After evolution, we measure changes in killing ability, toxin sensitivity and resource competitive ability to test for signs of coevolution between killer and sensitive strain, as well as between a killer host and its virus. Our analyses indicate the rapid coevolution between killer and sensitive strain, induced by the appearance of toxin-resistant mutants, causing an initial increase followed by a decrease in killing ability. Simultaneously, coevolutionary changes happen in host and killer virus. Changes in killing rate are constrained by a trade-off with resource competitive ability in the absence of sensitive cells, while they correlate with fitness in the presence of sensitive cells. These results show the potential for coevolution in the yeast killer system and support our previous findings of co-adaption between host and virus in wild yeast killer strains (chapter 4 of this thesis).

Materials and Methods

(a) Strains and media

We used previously constructed K1 laboratory killer and isogenic sensitive strains, each containing different selective markers (ho::kanMX4/ho::natMX4, causing geneticin and nourseothricin resistance and ho::hphMX4, causing hygromycin B resistance) in the BY background (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 consecutive transfers and for the competition experiments to estimate fitness. YPD agar supplemented with appropriate antibiotics (geneticin: 0.2mg/ml; nourseothricin: 0.1mg/ml; hygromycin B: 0.3mg/ml) was used during weekly transfers where killer and sensitive strains were separated and reset at 1:10 ratio and to estimate colony-forming units of both strains in competition assays. Assays of killing rate, 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 fitness 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 for colonies with uracil prototrophy.

(b) Experimental coevolution

Five different experimental treatments were prepared each represented by three replicate populations (15 populations in total): (1) coevolution, mixed populations where both K and S were allowed to evolve, (2) asymmetric coevolution of K, mixed populations where only K was allowed to evolve and S was replaced from unevolved freezer stock every week, (3) asymmetric coevolution of S, mixed populations where only S was allowed to evolve and K was replaced weekly from unevolved freezer stock, (4) control K, monoculture of K, and (5) control S, monoculture of S. To start coevolution (1), stationary phase cultures of K and S strains were mixed at a ratio of 1:10 (based on measurements of cell densities) and approximately 10^6 cells of each mixture was evenly spread 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 1% was spread onto fresh agar medium. Since the K and S strains had different markers, after four 24-hour transfers (~25 generations), K and S cells were separated by plating 1% of the wash volume on YPD agar with appropriate antibiotics. After three days of incubation, populations were re-set to the initial 1:10 ratio by measuring K and S cell densities and mixing appropriate volumes to continue coevolution. To start the asymmetric coevolution treatments (2) and (3), stationary-phase cultures of K and S were inoculated on low-pH YPD agar at a ratio of 1:10 and 1% transferred to fresh medium every day. During the 4^{th} transfer, washed-off cells were plated on antibiotic agar, and only cells from one of the two competitors were mixed with similarly pregrown using antibiotic medium cells from the freezer stock of the other competitor. Two control populations were employed, where K and S were grown separately and transferred to fresh medium every 24 h. Every 4^{th} transfer they were accordingly plated on antibiotic media, followed with the transfer to low-pH YPD agar after 3 days of incubation. All populations were transferred for 20 weeks or ~500 generations.

(c) Asymmetric coevolution of K at different cell densities

We performed another evolution experiment to test for the effect of cell density of K on changes in killing rate and competitive fitness. Here, we allowed only K to evolve in the presence of S at three K:S ratios (1:1, 10:1 and 100:1), which were all higher than the K:S ratio of 1:10 used in the main evolution experiment. Initially plates were seeded with 500µl of a 1000-fold dilution of the stationary phase culture of S cells (~10^5 cells), overlaid with a droplet of 50µl K 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 cell density was adjusted based on OD_{600}. At each transfer, S cells were pregrown to stationary phase from the freezer stock, and K and S cells were adjusted to the appropriate ratio. Five replicate populations at each K:S ratio were transferred this way for 25 transfers, which involved ~5.5 to ~8 generations per transfer for the high (100:1) and low (1:1) K density, respectively, leading to ~130-200 generations in total.

(d) Assay of killing rate K

Low-pH YPD supplied with 0.003% MB agar plates were inoculated with 200 µl of a 100-fold dilution of YPD stationary-phase culture of S cells (~$4x10^5$ cells per plate). After the plates dried up, 5 µl aliquots of undiluted (~$2x10^8$ cells/ml) overnight K culture were put on top of the S cells as local patches. The size of the halo produced around the K patch was measured after 72 h of incubation at 25°C. As the halo size was then transformed into a killing rate by the number of killed initially present S cells (estimated from the area of the zone of growth inhibition assuming the initial density of (~$8x10^3$ sensitive cells per cm^2) divided by the number of K cells initially present (~1 x 10^6) and by 72 h, that is, the time period during which the halo developed. Our conclusion that only the initially present K and S cells are involved in the halo development is likely incorrect, but the standardization of conditions and use of the same S strain across assays make these estimates a reliable measure of relative killing rate (chapter 3 of this thesis).

(e) Assay of toxin sensitivity S

Low-pH YPD supplied with 0.003% MB agar plates were inoculated with droplets of 50 µl of a 100-fold dilution of YPD stationary-phase culture of S cells to be tested (~10^5 cells per droplet). After the patches dried up, 5 µl aliquots of undiluted (~$2x10^8$ cells/ml) overnight ancestor K culture were put as small patches on top of the S tester patches. The size of the halo produced around the K patch was measured after 72 h of incubation at 25°C. Sensitivity of S was expressed the same way as the killing rate of K (but under slightly different conditions, i.e. higher cell density of S cells), as the number of killed S cells (estimated from the area of the zone of growth inhibition assuming the initial density of ~$6x10^3$ sensitive cells per cm^2) divided by the number of killer cells initially present (~1 x 10^6) and by 72 h**.**

(f) Sporulation of sensitive and resistant clones

10 random clones with the background of the sensitive strain from the ancestor, and coevolving population after 200 generations, and from an coevolving population after 500 generations were isolated, and sporulated using standard tetrad analysis. 10 tetrads from each clone were screened for toxin sensitivity.

(g) Assay of competitive fitness

Fitness of selected K and S isolates was measured in pairwise competition experiments (using a toxin-resistant strain as reference for K and a cured version of K for S) and expressed relative to the ancestor of that strain. Briefly, the procedure was as follows. One percent of a stationary phase culture (~2x 10^6 cells) of both competitors was grown for 24 hours on the low-pH YPD agar plates to acclimatize to experimental conditions. Cells were then washed off with 10 ml of water, mixed in equal volumetric proportion and 10 µl (~$2x10^5$ cells) was spread on a 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 fitness against the competitor was calculated as the ratio of their Malthusian parameters (Lenski et al. 1991), and normalized by a similar relative fitness of the ancestor. Competitions were performed with three or five-fold replication depending on the number of isolates assayed.

(h) Cross-infection of ancestral and evolved killer 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 x g), washed with SEKS buffer (1M sorbitol, 0.1M EDTA, 0.1 M Na2SO4, 0.8 M KCL, pH=7.5) and suspended in 10 ml PKE buffer (30 mM Na2HPO4 150 mM KCL, 10 mM EDTA, pH=7.6). Cells were treated with 0.1%-1% non-ionic detergent (Np40) and incubated for 1 hour at 30°C. Disrupted cells were centrifuged at 4,000 x 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 x 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 likely also the virus. To infect a novel host, exponentially growing host cells were collected by low-speed centrifugation (3000 x g) and washed four times with water. Cells were suspended in 1M LiAc and immediately collected by centrifugation at 13,000 x 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 8,000 x 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.

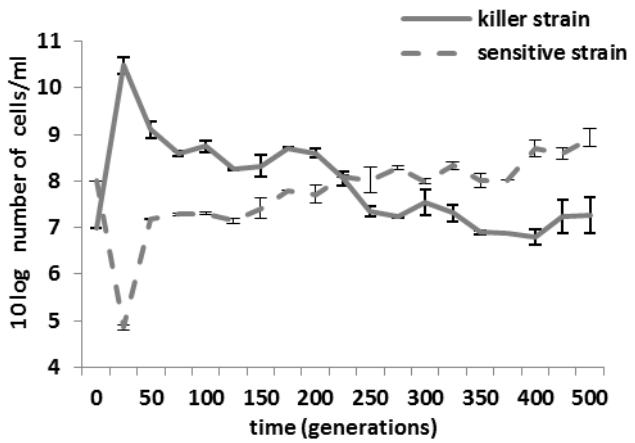
(i) Statistical analyses

We used pairwise comparisons based on two-sample *t-tests* (with unequal variance) for testing phenotypic changes across different experimental conditions. 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 tested replica population outcomes by two-sample *t-tests*. The density dependent effect of killing rate was tested by One-way ANOVA. Two-way ANOVA was used to test the effect on competitive fitness from the interaction between K:S ratio and presence/absence of sensitive cells. To test the trade-off between killing rate and resource competitive ability we used Pearson's correlation coefficient. Two-way ANOVA was used to test the effect of host and virus on killing rate from the cross-infection with foreign/own viruses.

Results

We allowed populations of two isogenic strains of *S. cerevisiae* (except for an antibiotic-resistance marker), one carrying a cytoplasmic toxin-producing killer virus K1 (K), the other without virus and hence sensitive to the toxin (S), to evolve on YPD low pH agar medium for 500 generations under three different conditions: 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 "unevolved" freezer stock, and monoculture control populations of K and S. The K:S ratio in the three mixed-population treatments were weekly reset by plating on selective medium and mixing them again at 1:10 ratio to ensure maximum opportunity for interaction, since K was expected to increase in frequency under these conditions (Wloch-Salamon et al. 2008). Figure 1 shows the frequency of evolving K and S in the mixed-population treatments at the end of each week before their ratio was reset. The rapid increase of K (and corresponding decline of S) in the first week (~25 generations) reflects the competitive superiority of K under these conditions. Note that despite resetting the K:S ratio to 1:10 at the start of every week, the weekly invasion of K and reduction of S declined in all treatments after the first week (see Fig. 1). The change in dynamics after the first week may be due to the growth-reducing effect of antibiotics (used to separate K and S after the initial week) on the other competitor (not carrying the resistance gene). However, the evolutionary conditions also affect the changes in invasion dynamics, as can be seen from the lower final densities of K when S is allowed to coevolve (Fig. 1a) relative to where S is not allowed to evolve (Fig. 1b; *t-test* on final density of K in coevolution versus asymmetric evolution: t= -3.01, df=4, 2-tailed P=0.039); final density of S does not differ between evolutionary conditions (Fig. 1a and 1c; t=1.76, df=4, 2-tailed P=0.152).

a)



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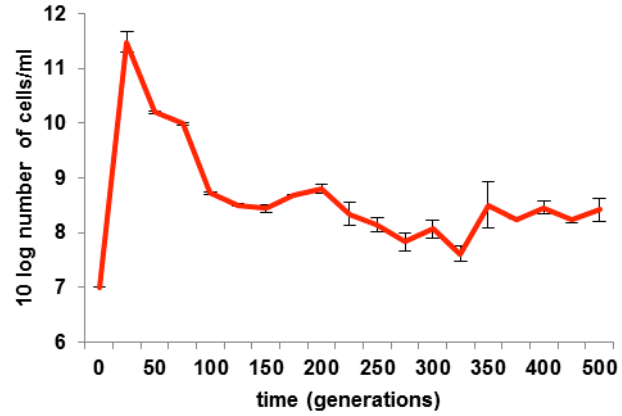

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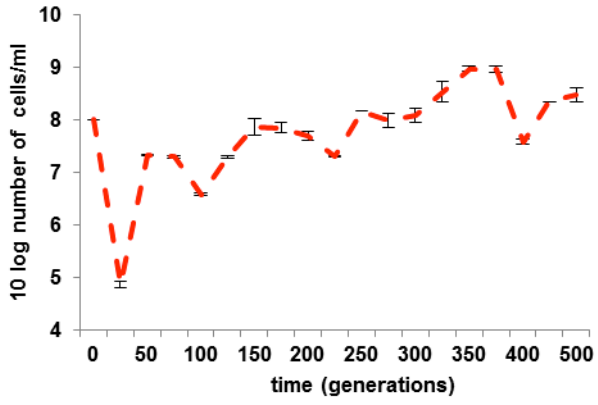
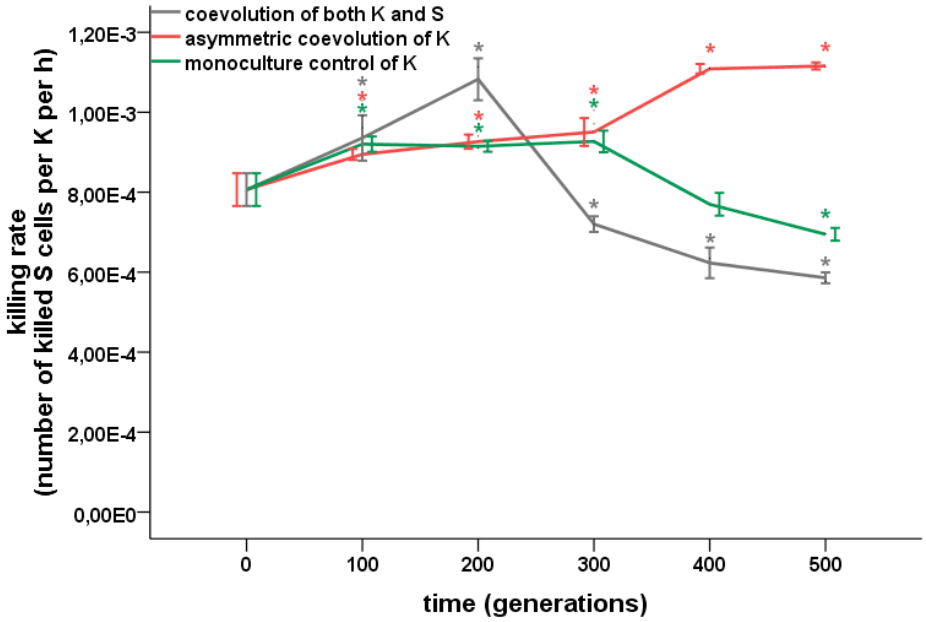


Figure 1. Average frequency (cell density per ml) of killer (K) and sensitive (S) cells in mixed populations during the 500 generations of evolution under three conditions: (a) coevolution of both K and S, (b) evolution of K only (with weekly replacement of S from "unevolved" freezer stock), and (c) evolution of S only (with weekly replacement of K from "unevolved" freezer stock). Shown are the frequencies before the weekly resetting of K:S to 1:10. Error bars represent standard errors of the mean based on the average of three replicate populations.

Changes in killing rate K and toxin sensitivity S

To measure changes in the killing rate of K and toxin sensitivity of S during evolution, 20 clones of both strains from each of the three replicate populations per treatment isolated at 100-generation intervals were assayed (summing up to 1840 assayed clones, including the ancestors). Fig. 2 shows changes in the average killing rate and sensitivity across conditions and evolutionary time. To compare dynamics across conditions, we used two-sample *t-tests* (with unequal variance) to test for significant changes in killing rate and toxin sensitivity relative to those of the ancestor using the different values for each of the three replicate populations. Asterisks in Fig. 2 indicate significant changes relative to the ancestor after serial-Bonferroni correction (Rice 1989) per phenotype. In the absence of S, killing rate slightly increases, followed by a decrease. The evolutionary dynamics are more conspicuous in the mixed populations, where K evolves clearly increased killing rates when S is not allowed to evolve, while K evolves initially even higher increases in killing rate followed by a stark decline when S is allowed to coevolve (Fig. 2a). More uniform dynamics are seen for toxin sensitivity of S (Fig. 2b). Here, in the absence of K, sensitivity increases monotonically – presumably as a correlate of increased resource competitive ability (see below). When K is present, toxin sensitivity decreases in both mixed treatments to similar final levels (zero in the coevolving populations), although it happens much faster when K is also allowed to evolve (2-tailed $P<0.01$ for *t-tests* comparing these two treatments at 100, 200, 300 and 400 generations). The deviating dynamics in killing rate and toxin sensitivity observed when both competitors are allowed to coevolve, suggest that changes in both K and S are involved in causing these dynamics.

a)



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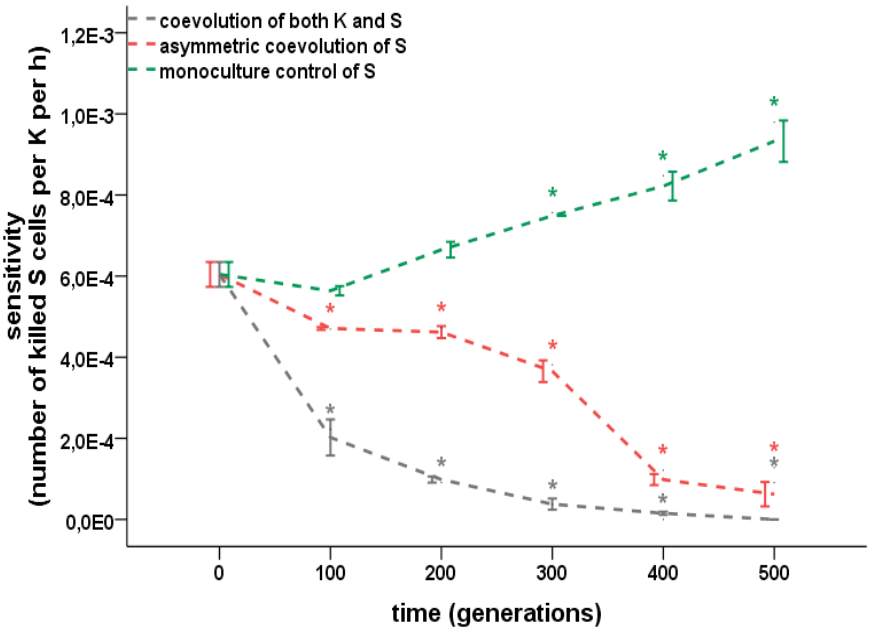

Figure 2. Changes in the killing rate of K (a) and toxin sensitivity of S (b) under different experimental conditions. Estimates are derived from measurements of 20 clones from each time point and replicate population. Errors bars represent standard errors of the mean based on the average of three replicate populations. Asterisks indicate significant changes relative to the ancestor based on pairwise comparisons corrected for multiple testing.

Evolution of toxin resistance

The rapid loss of toxin sensitivity in the coevolving populations (Fig. 2b) suggests that it arose by a single mutation. To examine this possibility, we plotted the frequency of S clones with complete toxin resistance (i.e. the complete absence of a "halo") for all time points and conditions (Fig. 3). Whereas not a single tested S clone was resistant in the control populations, the frequency of resistant clones is an inverse mirror image of toxin sensitivity (see Fig. 2b) for the two mixed-population treatments. This indicates that the distribution of toxin sensitivity is bimodal with peaks at zero and ancestral sensitivity, implying that the loss of sensitivity indeed involved a single-step process: the occurrence and selection of resistant mutants. The frequency of resistant mutants increased faster when K was allowed to coevolve than when K was weekly replaced by the ancestral strain (tested differences in the time at which resistant clones had a 50% frequency, estimated from fitted logistic model for each replica population: t=-14.88, df=4, 2-tailed P<0.0001).

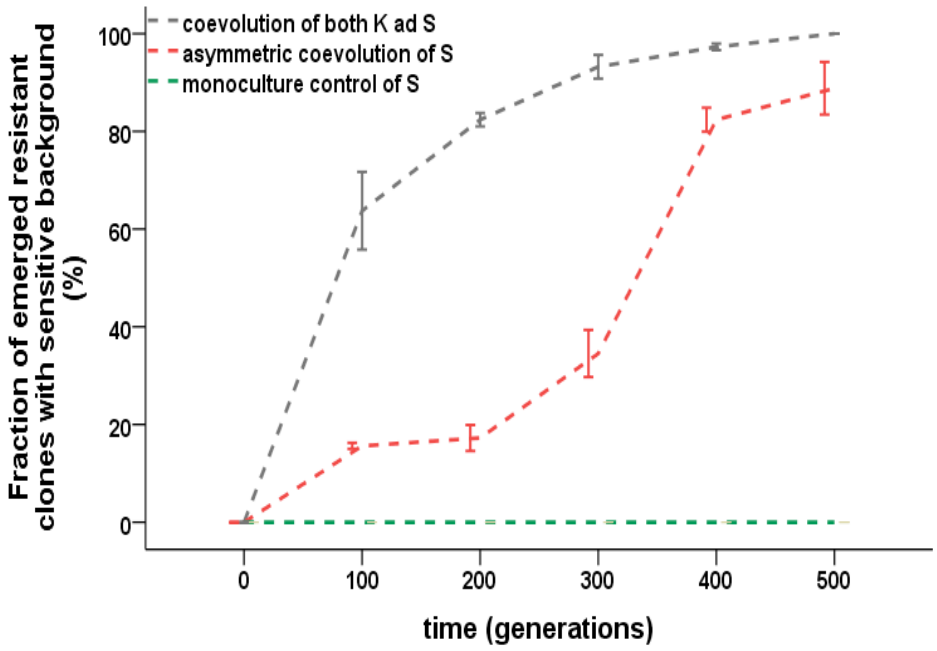

Figure 3. Average frequency of *de novo* evolved fully toxin-resistant mutants in the background of the sensitive strain in the two mixed-population treatments. Errors bars represent standard errors of the mean based on estimates for the three replicate populations per treatment.

To verify the suggestion that resistance involved a single mutation, we sporulated 10 S clones from various time points and phenotypes to look at the segregation of resistance phenotype in the tetrads of these diploid cells: the toxin-sensitive ancestor as a control, toxin-sensitive clones from the three coevolving populations after 200-generations when resistance had spread, and fully resistant clones from the final time point (500 generations). At least 10 tetrads from each segregated clone (100 in total) were screened for patterns of segregation and resistance phenotype. The 500-generation resistant clones showed a surprising segregation pattern, where always two of the four spores in the tetrad were inviable and the other two were fully resistant, suggesting that the resistance mutation had an associated meiotic-drive like phenotype (i.e. killing haploid spores without this mutation). As expected, the S ancestor showed normal segregation of four sensitive spores. However, the S clones from generation 200 showed a diverse picture, with some showing the segregation of four sensitive spores, while others had two viable toxin-sensitive spores and two unviable spores. Together these results imply that the evolution of toxin resistance was a two-step process, where the first step was the selection of a meiotic drive mutation presumably showing heterosis (i.e. a fitness benefit in heterozygotes), followed by a mutation causing toxin resistance which is linked to the first mutation. The fact that all tetrads from the 500-generation clones showed the correlated segregation of meiotic drive and resistance mutation justifies that they are linked.

To partially test this hypothesis, we performed competition assays involving toxin-sensitive clones with and without the lethal mutation and fully-resistant clones to measure their relative fitness (Fig. 4). These assays showed that the lethal mutation causes an almost 30% competitive benefit in heterozygotes, confirming the heterosis effect of the meiotic drive mutation, while the double mutant carrying both lethal and resistant mutation has lower fitness (although still higher than the ancestor). Sensitive clones without the lethal mutation showed only a marginal fitness increase, suggesting that they had undergone little or no change yet. In sum, the evolution of toxin resistance was a two-step process, where the first (meiotic drive) mutation provided a large resource competitive benefit, which prepared (in not understood ways) for the second mutation causing toxin resistance, which was driven by an interference competitive benefit.

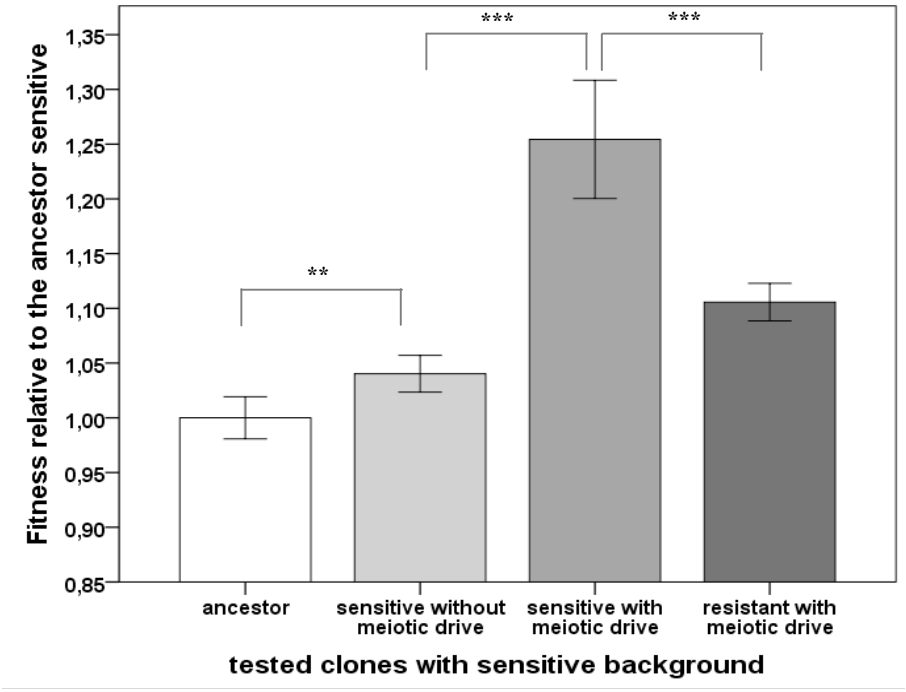

Figure 4. Relative fitness of the sensitive ancestral strain, a sensitive clone from generation 200 without the meiotic drive mutation (see text), a sensitive clone from generation 200 with the meiotic drive mutation, and a fully resistant clone from generation 500 (also carrying the meiotic drive mutation). Error bars represent standard errors of the mean based on ten replicate assays. Asterisks indicate significant differences in pairwise comparison (*p<0.05, **p<0.01, ***p<0.0001).

We also asked whether the appearance of resistance was influenced by the frequency of the killers. For that reason we employed additional short-term coevolution experiments using 1:10 and two new killer-sensitive ratios, 1:1 and 1:100. We screened three replicate populations during each transfer for the presence of resistant clones by plating on selective media and analysing single clones for toxin sensitivity. We found that when frequencies of competitors were equal, resistance could not develop due to the rapid elimination of sensitive cells (which were lost after two transfers). For 1:100 ratio, we found the emergence of resistance, although it appeared and became fixed later than at the previously used 1:10 ratio, indicating that evolution of the toxin resistance depends critically on the frequency of interaction with killer cells.

Evolution of killing rate and fitness

Next, we sought to understand the evolutionary changes observed for the killing rate. We first measured the fitness consequences of killing rate changes in the absence of killing benefits (i.e. in the absence of S) for all three evolutionary conditions (Figure 5). All pairwise comparisons of mean killing rate (Fig. 5a) and mean relative fitness (Fig. 5b) between ancestor and 500-generation evolved clones were significant (two-tailed P<0.0001 for killing rate and P<0.01 for fitness using *t-tests*). Three conclusions emerged. First, the decline of

killing rate observed in the absence of S (control populations) was associated with the largest increase in fitness, consistent with resource competitive benefits driving this change. Second, the largest increase in killing rate, evolved in the asymmetrically evolved K populations, corresponded to the smallest increase in fitness, suggesting that increased interference competitive ability drove this change. Third, the large decrease in killing rate in the coevolving populations was associated with a ~15% increase in fitness (almost equal to that of the control populations t= -1.65, df=4, 2-tailed P=0.174), suggesting that, despite the initial increase in killing rate (Fig. 2), the final genotypes are similar as those selected without S. Together, these changes suggest that a trade-off between killing ability and competitive fitness exists: evolution leads either to superior resource competitors (in the absence of S) or to superior interference competitors (in the presence of S), but not both.

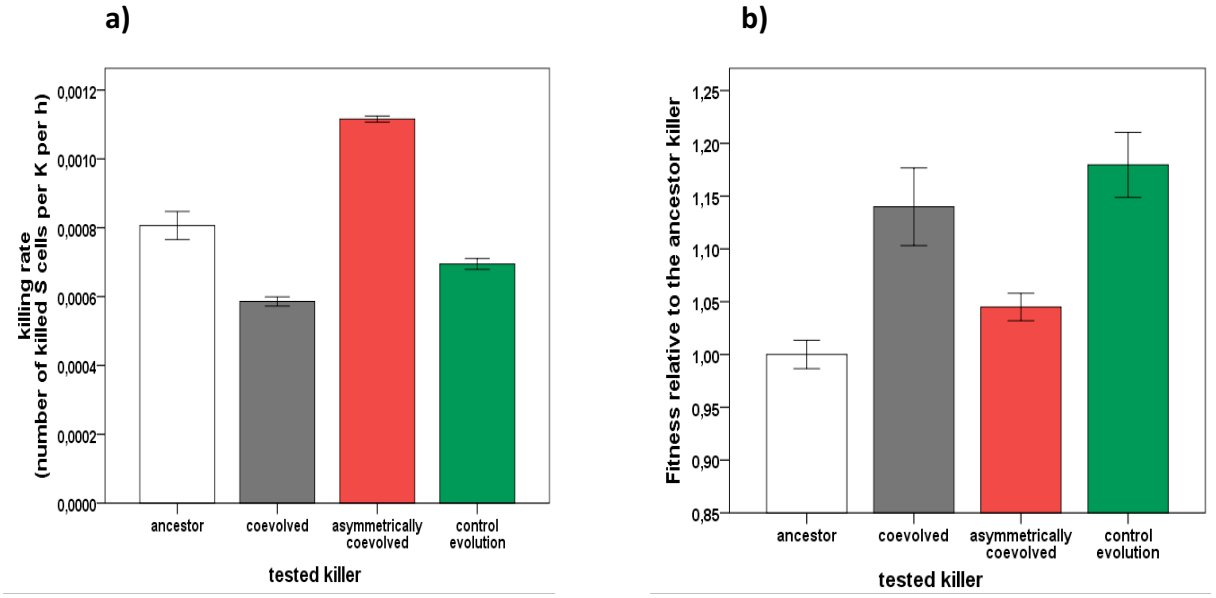

Figure 5. Average killing rate (a) and relative fitness (b) of the ancestral and 500-generations evolved K clones in three experimental treatments: coevolution of K and S, asymmetric coevolution (i.e. evolution of K with replacement of S) and control evolution (i.e. monoculture). Fitness of K is measured in the absence of sensitive cells. Error bars represent standard errors of the mean based on the mean estimates for the three clones of the ancestor and three replicate evolved populations per condition.

To better understand the relationship between killing ability and fitness, we selected a number of additional K genotypes from the evolved populations in which we found an especially high increase in killing rate in the previous experiment, i.e. in the presence of S but without the ability of S to evolve. We made an attempt to select for even higher killing rates by increasing the density of K relative to S (using 1:1, 10:1 and 100:1 relative K:S cell densities), by concentrating K cells on a single patch surrounded by S cells and transferring K cells from the edge of the patch after incubation during 25 cycles (equivalent to ~130-200

generations) in five replicate populations each (see Methods for details). Despite the shorter duration of evolution, killing rates increased beyond the killing rate observed in asymmetrically evolved K populations of the previous experiment (Fig. 6a). Moreover, initial density had a significant positive effect on killing rate (One-way ANOVA: F=67.29, dfs=3,14, P<0.0001). Thus, increased local density of K cells causes increased evolution of killing ability in the presence of S cells, probably caused by the larger mutation supply rate associated with the larger population size of K.

We then measured changes in competitive fitness of evolved K against a toxin-resistant reference strain under two conditions: (i) in the absence of S, where the resource competitive consequences are shown, and (ii) in the presence of S, where the combined effects of changes in resource and interference competitive ability are shown (Fig. 6b). Consistent with our previous results suggesting a trade-off with resource competitive ability (Fig. 5), we found significant reductions of resource competitive ability in the absence of S together with increased killing abilities (two-tailed P<0.05 using pairwise comparisons against the ancestor). However, when S was present, we found increases in relative fitness (two-tailed P<0.01 using pairwise comparisons), except for the ancestor. This clear effect of S on competitive fitness explains the evolution of increased killing rates observed in populations where S was present (Fig. 5a and 6a). 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).

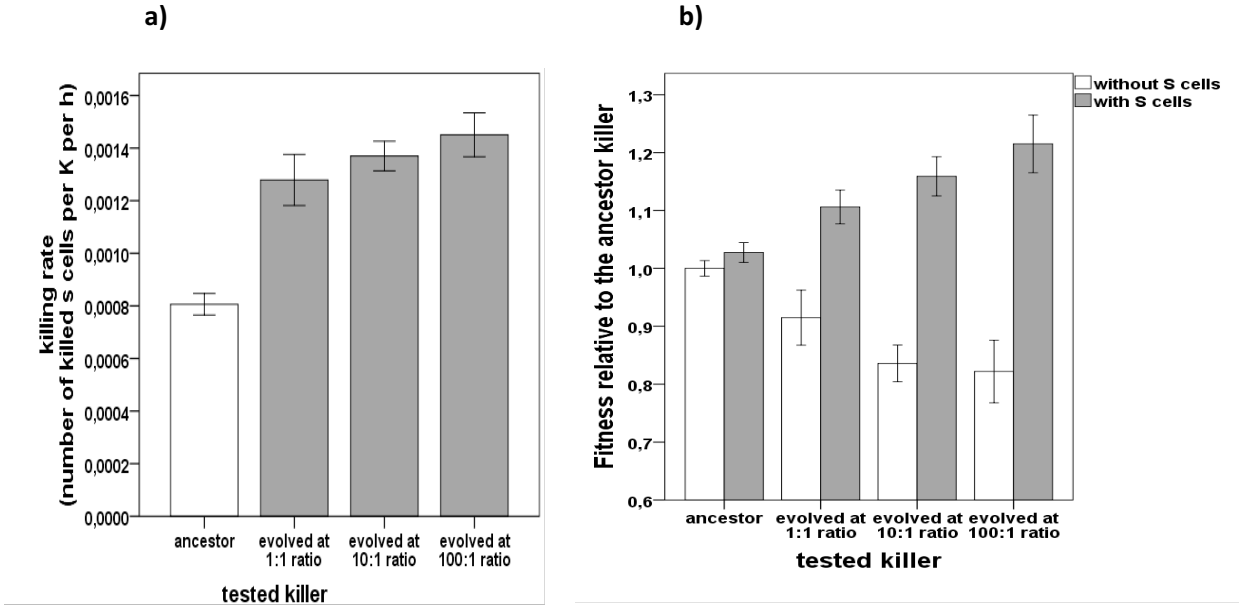

Figure 6. Average killing rate (a) and relative fitness (b) of K isolates (mixed population samples) evolved in a separate experiment (see Methods), where they were growing together with S on agar medium for ~130-200 generations at three initial K:S density ratios (1:1, 10:1 and 100:1). Fitness was measured in the absence and presence of sensitive cells against a resistant reference strain and expressed relative to the ancestor. Error bars represent standard errors of the mean based on five replicate assays.

To formally test for a trade-off between killing ability and resource competitive ability we pooled all estimates for the ancestor and evolved K strains (using the average per treatment). As can be seen in Fig. 7, the combined data indicate a strong trade-off (Pearson's *r*=-0.914, n=7, *P*=0.004). Increased killing rates are strongly associated with higher metabolic costs, which may only turn into selective benefits when killing toxin-sensitive cells provide compensating benefits by monopolizing (and possibly even freeing) limiting resources.

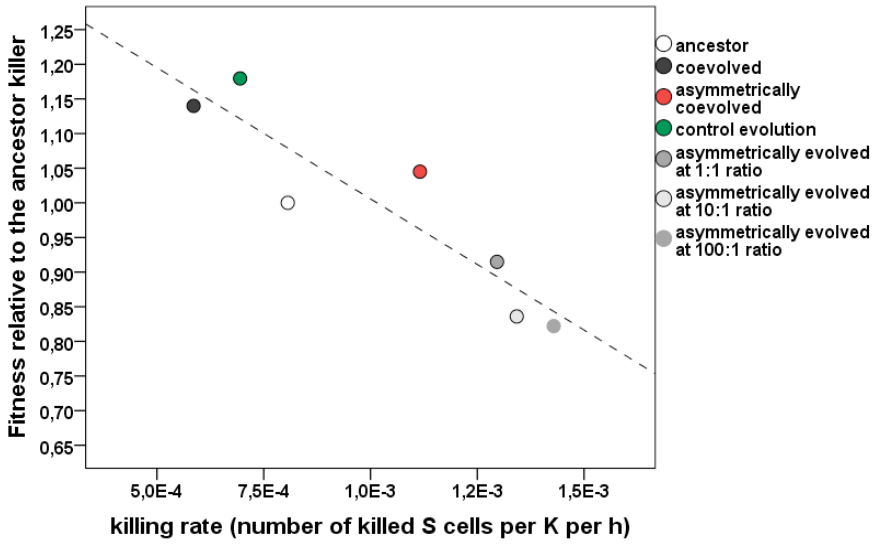

Figure 7. Relationship between the mean killing rate and relative fitness of ancestral and evolved K strains from all experimental treatments (coevolution, asymmetric coevolution, control evolution and asymmetric evolution at three ratios; *r*=-0.914, n=7, *P*=0.004).

Tests of host-virus coadaptation

Finally, to test whether coevolution happened not only between K and S hosts, but also between host and killer virus, we isolated the virus from the K ancestor and the strain with the highest evolved killing rate (i.e., evolved at a 100:1 K:S density ratio). The two viruses were used to re-infect cured versions of the two hosts. All four host-virus combinations were then assayed for killing rate (Fig. 8). Note that performance of the two (ancestral and evolved) original host-virus combinations is slightly lower than before curing and transfection (Fig. 6a), which is probably due to suboptimal viral titres (chapter 4); however, results are comparable across the four combinations, since all measurements were made immediately after transfection. Analysis of variance (Table 1) indicates that changes in both host and virus contributed to the increased killing rate of the evolved K. 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. ANOVA of the effect of host and virus on killing rate from the cross-infection of killer viruses between ancestor and 100:1 K:S ratio-evolved K. Mean Square is multiplied by 1,000 for killing rate.

Source	df	Mean Square	*F*	*P*
Host	1	0.289	110.687	<0.0001
Virus	1	0.42	15.913	0.004
Host x virus	1	0.637	243.901	<0.0001
Residual	8	0.003		

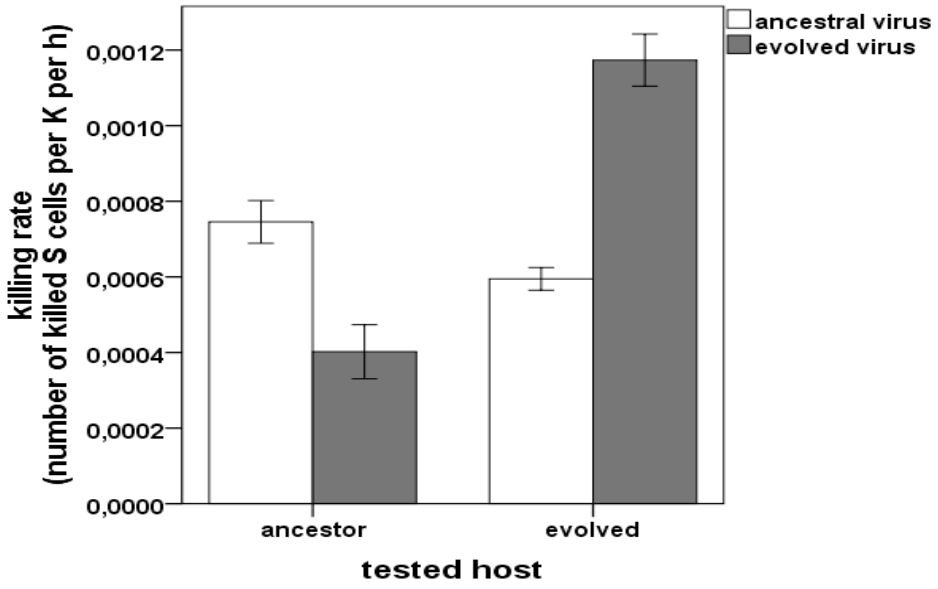

Figure 8. Effect on killing rate of cross-infecting viruses between ancestral strain and host clone isolated from the evolution at 100:1 K:S ratio treatment. Error bars represent standard errors of the mean based on the mean estimates for three replicates.

Discussion

We demonstrated the remarkably rapid and parallel coevolution in mixed populations of two strains of *S. cerevisiae*, one carrying K1 killer virus encoding an anti-competitor toxin, the other an isogenic toxin-sensitive strain without virus, at two distinct levels: between killer and sensitive strain and between host and killer virus. At the level of interactions between killer and sensitive strain, we observed the rapid invasion of toxin-resistant mutants derived from the sensitive strain. The rise of resistance initially accelerated the evolution of increased killing ability of the killer strain, but later – when the frequency of sensitive cells dropped below a critical value – caused selection for decreased killing ability. By comparing results obtained for the coevolving populations with results obtained under conditions where the competitor was not allowed to evolve or was absent altogether, we could show that these changes were due to reciprocal evolutionary changes in both strains –the hallmark of coevolution. At the level of host-virus interactions, swapping the virus between the ancestor and an evolved strain with high killing ability revealed that changes in both host and virus contributed to the increased killing ability, and that these changes were positive only when combined – supporting that these changes were reciprocally triggered, indicating co-adaptation also at this level.

The rapid co-evolution between host and virus resulting in host dependence, which we observed here, is consistent with the dependence of yeast hosts on their native killer viruses among natural isolates observed in our analyses of 136 yeast strains (chapter 4). It implies that host-virus associations in natural killer strains are not necessarily very old, even when they show signs of host dependence. On the other hand 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 co-adaptation.

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), but often without information about the dynamics and reciprocal nature of the process. Short time-scale co-evolutionary 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 difference between our study and the bacteria-phage experiments is that the latter involved antagonistic interactions (Stenseth and Smith 1984), because negative effects on the host due to phage adaptation do not affect phage fitness as much as it does killer virus in yeast. This is because bacterial phages can escape their host and infect other hosts, whereas killer viruses cannot escape their host except in rare sexual crosses (lack of horizontal transmission). A related study to ours looked at the co-adaptation between the nuclear and mitochondrial genome in experimentally evolved yeast populations (Zeyl et al. 2005). By swapping mitochondria between

ancestor and evolved cells, it was recorded that the fitness was increased by mutations in both genomes. However, whereas we found signs of "addiction" of evolved host and virus at the level of killing ability (Fig. 8), the fitness effects of evolved nuclei and mitochondria were more or less additive. Another related study by Bouma and Lenski (1988) reported signs of addiction in a bacterium-plasmid association. They evolved bacteria carrying a plasmid with an antibiotic resistance gene, and found fitness reductions from removing the evolved plasmid, whereas the original plasmid carried a fitness cost in the ancestral strain.

A significant contribution from our study was the clear-cut support for a trade-off between killing ability and resource competitive ability (i.e. fitness in the absence of sensitive cells; see Fig. 7). Fitness trade-offs among traits are important for understanding variation among species, in particular the divergence between specialized competitive strategies (Stearns 1989; Duffy et al. 2007). Similar 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) due to the energy investments in their production and viral or plasmid carriage. By comparing competitive fitness of strains with varying killing ability in the absence and in the presence of sensitive cells, we verified that the negative correlation between killing ability and fitness became positive when interference competitive ability contributed to fitness. The fitness costs associated with killing ability stimulated the evolution of specialisation towards either increased killing ability when conditions allow (e.g. low dispersal, frequent interactions with sensitive cells and a high local density of toxin producers) or increased resource competitive ability when toxic killing does not provide benefits, instead of generalists that are good in both (Brockhurst and Koskella 2013).

One of the most striking findings was the rapid emergence and invasion of toxin resistant mutants derived from the toxin-sensitive strain, which seemed to involve two independent mutations: a meiotic drive mutation conferring a fitness advantage in heterozygotes, followed by a mutation causing toxin resistance and associated with lower fitness. Halo assays of 100 tetrads from various populations indicated that resistance occurred in a single step, but the fact that we found no resistance (among 30 tetrads) without the meiotic drive phenotype strongly suggests that the selection of the resistance mutation depended on the presence of the meiotic driver mutation. The fact that resistance and meiotic drive phenotype co-segregated in all resistant cells that were tested suggests that both mutations are physically linked or even represent intermediate and final confirmation of a single, complex mutation. Interestingly, meiotic drive mutations with a fitness advantage in heterozygous state have been found before in Drosophila (Mukai and Burdick 1959), and meiotic drive elements associated with prions were observed before in the fungus *Podospora anserina* (Dalstra et al. 2003), as well as in yeast (Krishnan and Lindquist 2005). Whether these meiotic drive mutants conferred toxin resistance is unknown.

To summarize, we identified the rapid parallel coevolution between a yeast strain and a K1 killer virus, and between this killer strain and a sensitive strain, during 500 generations of evolution in the

laboratory. Remarkably, coevolution between killer and sensitive strain co-occurred with coevolution within the killer strain between yeast host and killer virus. However, we cannot disentangle cause and consequence, and determine whether killer-sensitive coevolution affected killer-virus adaptive responses, or vice versa. To understand this in more detail, analyses of host-virus coevolution of evolved killers also from the other treatments (i.e. monoculture, and mixed with non-evolving sensitive strain) would be informative. We could then test whether larger coevolutionary responses between yeast and killer virus occurred when parallel killer-sensitive coevolution took place, because changes in one interaction stimulated coevolutionary responses in the other. Additionally, to fully understand the mechanisms and reciprocal nature of the observed coevolution, genomic analyses of sensitive and killer strains, including its viral RNA, are needed. We intend to perform such analyses in the near future.

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Chapter 6

General discussion

Introduction

The killer phenotype is still a small subject of study in the continuously expanding field of yeast research. This is a shame as, in fact, yeast killer strains, carrying a pair of protein-coated dsRNA viruses which provide the host with a toxin and immunity to it, offer a rare and excellent model system for studying both the evolution of interference competition as well as host-virus coevolution. The stability of the killer phenotype suggests that carrying a killer-virus is generally beneficial to the host. However, it remains poorly known what selection forces are actually at work and what can be their beneficial effect on the host and virus genomes. This thesis aimed to both increase the knowledge on the general biology and biodiversity of the yeast-virus system as well as the evolutionary mechanisms that have shaped it.

Ecological and evolutionary role of the killer phenotype

The first step of this thesis was to assess how prevalent killer viruses actually are in two collections of yeast isolates. Before this thesis available data were mostly anecdotal. It is believed that killer viruses are very common based on the fact that they can be found among yeasts isolated from different sources, including fruits, mushrooms, fermenting materials, soil, and decaying plants. In these and other environments, they might play a significant role in the ecology of yeast communities (Ganter and Starmer 1992; Pintar and Starmer 2003). In recent years, protocols of yeast isolation have been improved, so that sampling became more representative of wild yeasts populations. Even more importantly, whole genome sequencing provided increasingly detailed insights into the phylogenetic relatedness among sampled strains so that sub-sampling of the same variety/genotypes could be prevented.

In **chapter 2** I have made good use of these advances to provide the first comprehensive estimate of the frequency of yeast killer strains in diverse habitats. I used two previously described *Saccharomyces* collections (136 strains) including isolates of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* derived from various habitats and geographical regions. The genome sequences of all strains had been analysed before and, therefore, their relatedness was unequivocally known (Liti et al. 2009; Schacherer et al. 2009). I assayed them, using the "Halo method" (Kishida et al. 1996), for the presence of killer-type toxicity and resistance to it. I found that a relatively small fraction (~10.3%) of the strains contained a virus producing a toxin of a known type (K1, K2 or K28) (Schmitt and Breinig 2006; McBride et al. 2013), while a much larger fraction (25%) was fully resistant to at least one of these three killer toxins (12.5% to two and ~9% to all three toxins). Thus, viral infections are not very frequent. On the other hand, killer toxins seem to have affected various yeast communities, since about a quarter of the isolates exhibited resistance that was

most likely chromosomally coded. This piece of information agreed with the results of my laboratory coevolution experiment (**chapter 5**). By using a constructed K1 killer and an isogenic sensitive strain, I was able to allow them to interact for 500 generations. Under conditions resulting in low dispersal (promoting the evolution of toxin producers), I identified the prompt appearance of resistant mutants within the sensitive strain. Resistance quickly spread over the whole population, indicating that selection favoured it in the presence of the killer strain. It is known that sensitive strains can become toxin-resistant by acquiring chromosomal mutations in genes involved in the structure and biosynthesis of yeast cell-wall components (Schmitt and Breinig 2002). Among the toxin resistant mutants that were identified before are strains with mutations in *kre* for the K1 toxin (Boone et al. 1990) and in *mnn* for the K28 toxin (Schmitt and Radler 1990). Other studies found a number of genes implicated in increased toxin resistance involved in mitochondrial, respiratory and ATP metabolism (Pagé et al. 2003; McBride et al. 2013). However, the exact mechanisms of toxin resistance and the conditions favouring them remain largely unknown.

Toxin producers pay costs of carrying toxin producing viruses. More precisely, they have lower resource competitive ability than non-producing and toxin-resistant strains (Wloch-Salamon et al. 2008). When sensitive cells are present but competitors are constantly mixed, killers get compensated only when present at a high frequency; at a low frequency killers are promptly eliminated from the population due to insufficient toxin concentrations and thus ineffective killing. When dispersal is limited, toxin producers benefit independently of their frequency, because toxin accumulates locally at concentrations sufficient to kill surrounding non-producers (Chao and Levin 1981; Greig and Travisano 2008). Under conditions where toxin producers do not benefit from carrying viruses, they are expected to lose them. What I observed in **chapter 2** is the presence of three kinds of yeast competitors: killer, sensitive, and resistant ones. This natural coexistence of killer, toxin-resistant and toxin-sensitive strains suggests that they may have evolved through reciprocal adaptations, generating biodiversity within wild yeast populations. Theoretical models have shown that their coexistence may result from trade-offs between competitive benefits and metabolic costs which are different for killer, resistant and sensitive strain, and result in dynamically balanced abilities to compete for resources and to interfere with each other (Czárán et al. 2002; Brown et al. 2009; Hibbing et al. 2010). It is possible that initially there were mostly killers and sensitives in a local environment, with resistance subsequently arising in the sensitive background. The fact that attempts to cure the resistant isolates from their viruses typically did not remove resistance suggests that resistance resulted from chromosomal mutations. The high frequency of resistant strains indicates that encounters with toxin-producers are frequent, as it seems not very likely that resistance is a pleiotropic effect of some other adaptation. The relative high frequency of sensitive strains may have several causes. One explanation being that killers exerted selection pressure on sensitives leading to the emergence of resistance among the latter. Killers were then left with limited benefits of carrying viruses (low killing advantage) and simply got under the

selection to lose them and become sensitive. If so, some killers may have never interacted with the resistant mutants, rather gradually adapted to the presence of sensitive individuals. Another possibility could be that a single lineage of killers diversified into new genotypes which later spread and adapted to local habitats. Subsequently, some of them co-adapted with their viruses (all killers identified in this study) and some other lost their viruses (because costs overwhelmed benefits). While these changes ensue, sensitives are stimulated to develop resistance.

Despite the prediction from theoretical work that toxin-producers, resistant and sensitive strains should dynamically co-exist (Kerr et al. 2002; Czárán and Hoekstra 2003), I did not find such co-existence in the laboratory experiment described in **chapter 5**. I only observed coexistence of killer and sensitive, and killer and resistant strains. However, I constrained the frequency changes of strains by the regular re-setting of both strains to the initial 1:10 killer: sensitive ratio, which limited the opportunity for the evolution of coexistence of all three players. Furthermore, when the frequency of killers was very high (equal number of killer and sensitive competitors), resistance could not be selected due to prompt elimination of the sensitive subpopulation. Hence, only for certain frequencies of killer and sensitive strain there is the opportunity for resistance mutations to arise and become fixed, allowing possible coexistence (through negative frequency-dependent selection) of toxin producers, sensitives, and resistant competitors.

The genetic basis of toxin resistance was elucidated by tetrad analysis. I found that it was likely determined by two independent chromosomal mutations. A meiotic drive mutation appeared first and was highly beneficial itself, showing strong overdominance effect on resource competitive fitness. The second mutation was identified only in resistant clones and seemed to be dependent on the meiotic drive mutation, because it was never found in the absence of the meiotic drive mutation. It is possible that both the natural and the laboratory evolved resistant strains are associated with mutations in known resistance genes, such as those involved in the synthesis of toxin receptors (Schmitt and Breinig 2002; McBride et al. 2013). It would be particularly informative to identify the genetic cause of both the meiotic drive mutation, showing overdominance for fitness, and the mutation conferring resistance to the killer toxin.

Assessment of killing ability

Interference competition via the production of anti-competitor toxins is widespread among microorganisms. Theoretical and experimental work has been done to understand the ecological and evolutionary role of this form of microbial competition (Czárán et al. 2002; Kerr et al. 2002; Pintar and Starmer 2003; Gulbiniene et al. 2004; Wloch-Salamon et al. 2008). An essential parameter of models addressing the role of toxic killing is the efficiency of killing, expressed by the number of toxin-sensitive individuals that are killed by a single toxin producing cell during a defined time interval. In practice, estimates of killing efficiency are often based on

rather qualitative methods measuring the halo, or zone of growth inhibition of sensitive cells, surrounding a patch of toxin producers. The molecular mechanism of toxin production and killing are generally well described (Marquina et al. 2002; Schmitt and Breinig 2002, 2006). It is the ecology and evolution of these systems that require more research, for which reliable estimates of killing ability is an essential prerequisite.
I performed a systematic comparative analysis of four methods, including the Halo method and three quantitative liquid assays. The aim was to identify a convenient method that would be sensitive and reproducible (**chapter 3**). I tested these methods with a set of three yeast killer strains (K1, K2 and K28). In sum, I found the classical "Halo method" to be the most convenient (easy application and low cost) and reliable in quantifying the rate of toxic killing and in discriminating between different toxin-producing strains.

Host-virus coevolution

The evolution of a symbiotic relationship between previously independent partners is considered a "major evolutionary transition" (Szathmary and Smith 1995). It remains a challenge in evolutionary biology to understand the mechanisms involved in this process. Understanding the evolution and stability of the yeast-virus symbiosis is a prerequisite for understanding the ecological and evolutionary role of killer strains. Therefore, in **chapter 4**, I described experimental tests of the dependence of the yeast host strains on their killer viruses.

I performed cross-infections among seven wild and one laboratory killer strain, belonging to two different species, *S. cerevisiae* and *S. paradoxus,* but hosting the same K1 type virus. I was able to identify clear signs of host-virus co-adaptation based on higher killing ability and virus stability for native relative to novel combinations. Curing the host from its virus caused a decline in competitive fitness for all native, but an improvement for all novel host-virus combinations. This last observation suggests that metabolic cost of carrying the virus could turn into partial dependence of the host on the inhabiting virus. Presumably, compensatory evolution during the time of symbiosis had removed the initial fitness costs and made the natural killer strains "addicted" to their killer viruses.

To study the dynamics of coevolution between the host and its killer virus, and to assess whether the initial fitness costs of viral carriage could turn into an addiction at short time scales, I conducted laboratory evolution experiments with a constructed K1 killer and (isogenic) sensitive strain (**chapter 5**). For 130 generations under conditions maximizing the benefits of toxic killing, a laboratory K1 killer strain was allowed to evolve in the presence of sensitive cells, which themselves were not allowed to evolve. Swapping the killer virus of ancestor and evolved killer indicated that both the evolved host and virus contributed to the increased killing rate of the evolved killer phenotype, and that these changes were beneficial only in the

presence of each other. This mutual dependence of evolved hosts and virus shows that they were reciprocally triggered – the hallmark of genuine coevolution (Brockhurst and Koskella 2013). The rapid coevolution leading to addiction of the host to the killer virus is consistent with my findings of a similar addiction in the wild yeast killer isolates (**chapter 4**). These findings may be partially explained by the fact that when a symbiont fully depends on the host, as in the yeast-killer system where no horizontal transmission occurs (Wickner 1996) natural selection prevents antagonism from the endosymbiont (Szathmáry and Smith 1995). Furthermore, co-adaptation between the host and its virus removes the cost of virus carriage or even causes the co-dependence between symbionts causing so-called "addiction" (i.e. turning costs into benefits), which was previously reported for *Wolbachia* bacteria and an insect host (Pannebakker et al. 2007) and bacteria and their plasmid (Bouma and Lenski 1988). The fact that I found that addiction evolves so rapidly implies that the host-virus associations in the wild killer strains do not need to be very old.

Evolutionary dynamics of killing ability

Fitness trade-offs among traits are important for understanding the evolution and variation among species (Duffy et al. 2007). Trade-offs cause the evolution of specialisation and constraints the evolution of generalists (Coyne and Orr 2004; Brockhurst and Koskella 2013). Among several striking results, in **chapter 5** I report on a clear-cut trade-off between killing ability and resource competitive ability (i.e. fitness) across all killer strains evolved under different conditions and all derived from the same constructed K1 strain. This result agrees with previous studies reporting that the evolution of virulence via toxin production carries negative fitness consequences for their producers (Cascales et al. 2007). This trade-off poses an important constraint to the evolution of killer strains, forcing the divergence towards either increased resource competitive ability, in the absence of killing benefits (e.g. when no sensitive competitors are present), or increased interference competitive ability, when toxin production provides benefits.

Another striking result was the series of rapid reciprocal coevolutionary changes in killer and sensitive strains. The dynamics of evolutionary responses were shaped by the opportunity of both strains to adapt. I aimed to maximize the adaptive processes by stimulating evolution of the killing ability by applying low dispersal in a structured environment, ensuring high local concentrations of the produced toxin. This factor is considered crucial for the success of toxin producers (Chao and Levin 1981; Greig and Travisano 2008; Wloch-Salamon et al. 2008). In the course of coevolution, selection initially favoured an increase in the killing ability of toxin producers, to enable their faster invasion. The sensitive population responded adaptively to the high toxin concentration of the toxin through the emergence of toxin resistance. As

resistant mutations became fixed within the population, killing benefits declined and selection favoured killers with decreased killing ability associated with increased resource competitive ability.

Finally, I found that the negative impact of killing ability on resource competitive ability in the absence of sensitive individuals (i.e. in the absence of killing benefits) was compensated once sensitive cells were present and killing was beneficial.

Future work

We have identified reciprocal evolutionary changes – the hallmark of coevolution -- in the killing and sensitive competitors and in the intrinsic interactions between hosts and viruses. Whilst my findings are clear and consistent, they are based on phenotypic analyses and the interpretation and comprehension of my results is likely to benefit from the availability of genomic data. Genomic information for the natural and experimental yeast hosts and their killer viruses (**chapter 4 and 5**) can confirm the reciprocal nature of evolution and uncover the genes involved in it. I suspect that most likely the affected host genes belong to the MAK, KEX, SEC, SKI families, which are required for the efficient maintenance, replication and expression of the killer phenotype (Wickner 1992; McBride et al. 2013). However, at this stage, I cannot exclude that other genes that affect the host-virus association, such as genes involved in general metabolism, may also be involved. Identifying the genetic changes underlying the coevolution observed in my experiments has the potential to provide new insights into the mechanism of the killer phenotype's evolution, and the specific role of host and virus in these changes. Changes in the viral genome could code either for a higher number of viral particles, a higher expression of the toxin, or for different toxins. Data on virus' sequences could also reveal recombination between phylogenetically separated viruses. Genomic information would be particularly welcome to reveal the mechanism of toxin resistance observed during coevolution in the laboratory (**chapter 5**). Sequencing of the naturally occurring resistant strains (**chapter 2**) could reveal whether the two-step mutational pattern observed in the laboratory, involving an apparent meiotic drive mutation, occurs also in nature. In this case, I predict that families of genes associated with the cell wall biosynthesis, particularly toxin receptors, to be involved.

Moreover, excitingly, evolution experiments with cross-infected strains could identify signs of co-adaptation within newly created combinations. In this way, I could test how reproducible coevolution of the host-virus interaction is and how specific the emerging partnerships. It would be informative to compare evolutionary histories known from nature with those obtained experimentally.

Coexistence of killer, sensitive and resistant competitors is another interesting aspect of further research. It could provide the tool to test conditions required for the theoretical prediction that toxin producers, resistant and sensitive genotypes will co-exist in a dynamic equilibrium (Kerr et al. 2002; Czárán

and Hoekstra 2003). In the work reported in this thesis (i.e. **chapter 5**) I could not test this prediction, since the initial high increase in frequency of killers forced me to re-set the ratio of competitors which limited the likelihood for the evolution of coexistence to occur.

Finally, naturally co-adapted symbioses of hosts and viruses could be tested in different environments (i.e. synthetic medium with depleted resources or medium with used up resources), which would allow to unravel the mechanisms underpinning the fitness cost of carrying the killer virus. For instance, one can look for a correlation between viral presence in particular strains and the reduced lifespan of the latter, or estimate the rate of viral loss during starvation. To understand the long-term fate of yeast-virus symbiosis, it will be informative to test whether natural isolates carrying killer viruses differ from isolates without virus in terms of longevity, and whether possible effects on lifespan are more serious than effects on the growth rate.

The killer yeast system is a powerful and promising model system for diverse ecological and evolutionary questions, particularly on host-symbiont coevolution and interference competition. I hope that the studies presented in this thesis will motivate further work with this system on these and related topics.

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English summary

Saccharomyces cells occasionally carry cytoplasmic ds-RNA "killer" viruses coding for low-mass proteins, which upon secretion to the environment can kill related cells that do not carry the viral particles. Such killer viruses are not infectious, and can spread only through cell division and during mating. Three principal classes of *Saccharomyces* viruses (ScV-M1, ScV-M2 and ScV-M28) belonging to the *Totiviridae* family have been characterised, each capable of forming a specific anti-competitor toxin and corresponding antidote. Presumably, toxic killing provides competitive benefits to the yeast host. However, the ecological and evolutionary significance of toxin production remains poorly understood. For example, it is unknown where yeast killers occur and at what frequency, how evolvable killing ability is, whether it is constrained by possible trade-offs with resource competitive ability and how it is shaped by interactions with toxin-sensitive competitors. Also unknown is how stable yeast-virus symbioses are, and how coevolution between host and virus may affect this stability and the killing phenotype itself. It is believed that killer yeasts are common based on the fact that they have been found among yeasts isolated from different sources over several decades. In **chapter 2**, we assay two large yeast collections from diverse habitats, including nature and man-made habitats (in total 136 strains with known genome sequences), for killer phenotype and toxin resistance. We find that ~10.3% carry a killer virus, while about 25% are resistant to at least one of the three known killer toxins (12.5% to different combinations of two and ~9% to all three), most likely due to chromosomal mutations. Analyses of their evolutionary relationship indicate that host-virus associations are relatively short lived, whereas the relatively high frequency of resistance suggests that toxins have a substantial impact on yeast evolution.

In order to understand the ecological and evolutionary role of toxin production, it is essential to reliably assess the killing rate of toxin producers by measuring how many toxin-sensitive individuals are killed by a single toxin producer during a given time interval. To identify a convenient method with high sensitivity and reproducibility, in **chapter 3** we perform a systematic comparative analysis of four methods, including the conventional "Halo method" and three more quantitative liquid assays. We apply these methods to a set of three known yeast killer strains (K1, K2 and K28) and find that the easy applicable Halo method provides the most sensitive and reproducible killing rate estimates (with best discrimination between killer strains).

Understanding the evolution of the yeast-virus association is crucial for a full understanding of the ecological and evolutionary role of killer strains. In **chapter 4**, we present experimental tests of the strength of the dependence of yeast host strains on their killer viruses. We cross-infect several viruses among killer strains of the genus *Saccharomyces* – all expressing the K1-type toxin, and test native and new combinations for the strength of host-virus co-adaptation. We find explicit host-virus co-adaptation, because native yeasts hosts display the highest toxicity and highest stability of killer viruses relative to hosts carrying non-native

viruses. Even stronger, we find that curing these wild killer yeasts from their virus reduces their competitive fitness, despite initial fitness costs of viral carriage reported for constructed killer strains. These results demonstrate co-adaptation of host and virus in the natural killer strains resulting in their dependence on the killer virus. To explore the evolutionary costs and benefits of virus carriage and toxin production, and understand whether they are shaped by the coevolution between host and virus and the presence of toxin-sensitive competitors in the environment, we conduct a series of laboratory experiments where we manipulate the opportunity for coevolution (**chapter 5**). Analyses of killing ability, toxin sensitivity and fitness (i.e. resource competitive ability), show rapid reciprocal changes in killer and sensitive strain when coevolution is allowed, modulated by the rapid invasion of toxin-resistant mutants and subsequent reduction of killing ability. Remarkably, we find that the rapid invasion of toxin-resistant mutants involves two mutational steps, the first being a mutation showing a meiotic drive phenotype as well as a strong fitness benefit in heterozygotes, the second the resistance mutation. Shifts in the competitive fitness of evolved killer isolates with increased killing ability show a clear trade-off between killing rate and resource competitive ability, indicating that resource and interference competitive ability are alternative competitive strategies. Moreover, by cross-infecting the killer virus between the ancestral and an evolved strain, we are able to demonstrate the rapid co-adaptation between host and killer virus, supporting our previous findings of co-adaptive responses in wild yeast killers (**chapter 4**).

Our analyses are based on screens of natural isolates, laboratory evolution experiments and phenotypic analyses, complemented by classical genetics. To more fully understand the reciprocal nature and molecular mechanisms of adaptive responses, genome analyses are required. The motivation for such analyses and other follow-up studies are proposed in **chapter 6**. My studies show the usefulness of the killer yeast system to address questions related to interference competition and coevolution, which may proof valuable also given potential applications of killer yeasts in the fermentation industry.

Samenvatting

Sommige *Saccharomyces* cellen dragen cytoplasmatische dsRNA "*killer*" virussen bij zich, die coderen voor kleine eiwitten. Bij uitscheiding in de omgeving kunnen deze eiwitten verwante gistcellen, die de virusdeeltjes niet bij zich dragen, doden. Killer virussen zijn niet overdraagbaar en kunnen zich alleen verspreiden door celdeling en gedurende paring. Er zijn drie verschillende klassen van *Saccharomyces* virussen bekend (ScV-M1, ScV-M2 and ScV-M28), die allemaal behoren tot de *Totiviridae* familie en elk in staat zijn tot het vormen van een specifiek *anti-competitor* toxine en het bijbehorende tegengif. Het doden via uitscheiding van deze toxines biedt vermoedelijk een competitief voordeel aan de gist-gastheer. Het ecologische en evolutionaire belang van toxineproductie wordt echter onvoldoende begrepen. Het is bijvoorbeeld onbekend waar gist *killers* van nature voorkomen en met welke frequentie, hoe evolueerbaar het vermogen om te doden via uitscheiding van toxines is, of dit vermogen beperkt wordt door *trade-offs* met competitief vermogen wat betreft *resource* gebruik, en hoe het gevormd wordt door interacties met toxine-gevoelige concurrenten. Het is eveneens onbekend hoe stabiel gist-virus symbioses zijn en hoe coevolutie tussen gastheer en virus deze stabiliteit en het "killing" fenotype beïnvloedt.

Er wordt algemeen aangenomen dat *killer* gisten veel voorkomen op basis van het feit dat ze gevonden zijn in vele gisten die in de loop van tientallen jaren geïsoleerd zijn uit verschillende bronnen. In **hoofdstuk 2** testen we twee omvangrijke gistcollecties (in totaal 136 stammen met bekende genoomsequenties) uit zowel natuurlijke als kunstmatige habitats voor killer fenotype en toxine-resistentie. We vinden dat ~10% van de stammen een killervirus bij zich draagt, terwijl ~25% resistent is tegen ten minste één van de drie bekende killertoxines (12.5% tegen een subset en ~9% tegen alle drie). Analyses van hun evolutionaire relatie geven aan de gastheer-virus interacties van relatief korte duur zijn, terwijl de relatief hoge frequentie van resistentie suggereert dat toxines een substantiële impact hebben op gistevolutie.

Om de ecologische en evolutionaire rol van toxineproductie te begrijpen, is het essentieel om de *killing rate* van toxineproducenten betrouwbaar vast te stellen, door te meten hoeveel toxine-sensitieve individuen gedood worden door een enkel toxine gedurende een bepaald tijdsinterval. Om een werkbare methode met hoge gevoeligheid en reproduceerbaarheid te vinden, vergelijken we in **hoofdstuk 3** vier methoden systematisch met elkaar, waaronder de conventionele "halomethode" en drie meer kwantitatieve methoden in vloeibaar medium. We passen deze methoden toe op een set van drie bekende gist killerstammen (K1, K2 en K28) en vinden dat de eenvoudig toepasbare halomethode de meest gevoelige en reproduceerbare schatting geeft van de *killing rate* (en het beste onderscheid mogelijk maakt tussen *killer* stammen).

Het begrijpen van de gist-virus associatie is cruciaal voor een compleet begrip van de ecologische en evolutionaire rol van killerstammen. In **hoofdstuk 4** presenteren we experimentele testen van de mate waarin gistgastheren afhankelijk zijn van hun killervirussen. We kruis-infecteren killerstammen van het geslacht *Saccharomyces* – die allemaal het K1-type toxine tot expressie brengen – met hun verschillende virussen en testen door middel van het vergelijken van oorspronkelijke en nieuwe combinaties de sterkte van gastheer-virus co-adaptatie. We vinden duidelijke gastheer-virus co-adaptatie: originele gist-gastheren zijn het meest toxisch en killervirussen blijven het meest stabiel gehandhaafd in hun oorspronkelijke gastheer. Bovendien vinden we dat het verwijderen van het virus uit de wilde killergisten de competitieve fitness van de gastheer verlaagt; dit in tegenstelling tot de initiële fitnesskosten die geassocieerd zijn met het meedragen van het virus voor geconstrueerde killerstammen. Deze resultaten demonstreren co-adaptatie van gastheer en virus in de natuurlijke killerstammen, wat resulteert in gedeeltelijke afhankelijkheid van de gastheer van het killervirus.

Om de evolutionaire kosten en baten van het meedragen van het virus en toxineproductie te verkennen, en om te begrijpen of deze mede gevormd worden door de coevolutie tussen gastheer en virus en de aanwezigheid van toxinegevoelige concurrenten in de omgeving, voeren we een serie laboratoriumexperimenten uit waar we de mogelijkheden voor coevolutie manipuleren (**hoofdstuk 5**). Analyses van de *killing ability*, toxinegevoeligheid en fitness (d.w.z. competitief vermogen wat betreft bouwstoffengebruik) van geëvolueerde gisten laten snelle wederzijdse veranderingen zien in de killerstam en de gevoelige stam als de stammen kunnen coevolueren. Coevolutie wordt zichtbaar door de snelle invasie van toxine-resistente mutanten en de daaropvolgende reductie van *killing ability* in de killerstam. Opmerkelijk genoeg vinden we dat bij de snelle invasie van de toxine-resistente mutanten twee genetische veranderingen betrokken zijn, waarvan de eerste een mutatie is die *meiotic drive* vertoont en een groot fitnessvoordeel biedt in heterozygoten, en de tweede een resistentiemutatie. Veranderingen in de competitieve fitness van de geëvolueerde killerisolaten met verhoogde *killing ability* laten een duidelijke *trade-off* zien tussen *killing rate* en competitief vermogen wat betreft *resource* gebruik. Dit geeft aan dat competitie door efficiënter gebruik van grondstoffen en via directe interferentie alternatieve evolutionaire competitiestrategieën zijn. Bovendien demonstreren we door middel van kruis-infectie met het killervirus van de voorouder en een geëvolueerde stam de snelle co-adaptatie tussen gastheer en killervirus, wat onze eerdere resultaten betreffende co-adaptieve responsen in wilde killergisten (**hoofdstuk 4**) bevestigt.

Onze analyses zijn gebaseerd op *screens* van natuurlijke isolaten, laboratoriumexperimenten en fenotypische analyses, gecomplementeerd door klassieke genetica. Om de wederkerige natuur en moleculaire mechanismen van de adaptieve responsen beter te begrijpen, zijn genoomanalyses nodig. De motivatie voor zulke analyses en andere mogelijke *follow-up* studies worden besproken in **hoofdstuk 6**. Mijn studies tonen de bruikbaarheid van het killergist-systeem aan voor het beantwoorden van vragen over

interferentiecompetitie en coevolutie, hetgeen waardevol kan blijken gezien de potentiele toepassingen van killergisten in de fermentatie industrie.

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I arrived to Wageningen University sometime in the middle of my PhD journey. The initial plan for me was to stay only for one year, hoverer unexpected progress of my project, together with fruitful cooperation with my promotors and great atmosphere provided by people from Laboratory of Genetics prompted me to prolong my stay and accomplish the PhD thesis in Wageningen. It was an amazing, inspiring and certainly beneficial time for my professional and personal life. Therefore, I would like to express my gratitude towards those who brought me to this point and helped me make this possible.

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Big thanks go to my PhD and non-PhD colleagues form the Laboratory of Genetics. It was a very productive and fun time to work with each one of you. I wish you all the best with your future scientific career. I would like to acknowledge my research group: Florien, Jianhua, Tina, Alex, Claudio, Eric, Jelle for many hours of mutual support, complaining and insightful discussions in in the office and coffee areas. Although, I have not been with you through the whole course of my project, I cherish our scientific and non-scientific time and I am very glad that I got to know you all. Good luck with finishing your PhD projects and starting your next step of your future career. Also, I would like to send my appreciation to Florien and Vivian for being my paranimphs and helping me succeed on that "big day". Gabriella, you are an amazing person. I would like to thank you for being always around, for all your support and most importantly for becoming such a good friend!

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Magdalena

June 2015

Curriculum Vitae

Magdalena Dominika Pieczynska was born on the 20th of March 1984 in Katowice, Poland. In 2008 she accomplished the Biomedical Sciences undergraduate studies at the London Metropolitan University, United Kingdom. She undertook the final research project on "Transfection of Hep2G human cancer cells with plasmid obtained from transformed bacteria". In parallel, she completed and obtained diploma of Medical Analysis course from Medical Academy, Poland. Upon completing her undergraduate courses, she went for the Human Genetics Master course at the St' George's University of London, where her master research project under the supervision of Prof. Andrew Crosby was based on "Exclusion of 21 candidate genes within the pontocerebellar hypoplasia gene locus". Before she started her PhD she worked as a research assistant at Brighton and Sussex University Hospitals and Guy's and St Thomas' NHS Trust, United Kingdom (2009-2010). In 2010, she started her PhD at the Evolutionary Genetics Group, Jagiellonian University, Poland, which she continued at the Laboratory of Genetics, Wageningen University. Her PhD aimed at understanding the ecology, coevolution and genetics of virus-yeast ''killer system'' of *Saccharomyces cerevisiae* community. Her project entitled "Role of anti-competitor toxins in the origin and maintenance of diversity in *Saccharomyces* yeast microbial populations" was supervised by Prof. Arjan de Visser and Prof. Ryszard Korona and is the subject of this thesis.

Currently, Magdalena is working as a postdoctoral researcher in the Bertus Beaumont Lab, at the Department of Bionanoscience, Delft University of Technology. Her project examines how evolution incorporates incompatible components into the bacterial flagellar motor.

List of Publications

Pieczynska, M. D., J. Visser, and R. Korona. 2013. Incidence of symbiotic dsRNA 'killer' viruses in wild and domesticated yeast. FEMS Yeast Research 13:856-859.

Pieczynska, M. D., **R**. Korona, and J. Visser. Comparative analysis of methods to assess toxic killing ability in *Saccharomyces* killer strains. (Submitted to Mysocience).

Pieczynska, M. D., R. Korona and J. Visser,. Experimental tests of host-virus coevolution in natural killer yeast strains. (Submitted to Evolution).

Pieczynska, M. D., Wloch-Salamon, D., R. Korona, and J. Visser. Rapid multiple-level coevolutionary dynamics in experimental populations of yeast killer and non-killer strains. (In preparation).

Presentations

2011 Pieczynska, M. D., J. Visser, and R. Korona.
"Incidence and experimental evolution of *Saccharomyces cerevisiae* killer system".
"Individual and Groups" Discussion Meeting cum Workshop, Almora, Indie.

2012 Pieczynska, M. D., J. Visser, and R. Korona.
"Prevalence of symbiotic RNA viruses in feral *Saccharomyces cerevisiae*".
7th International Symbiosis Society Congress "The earth's vast symbiosphere", Krakow, Poland.

2013 Pieczynska, M. D., R. Korona and J. Visser.
"Signature of coevolution between virus killer and its host in yeast strains".
Gordon's Research Conference: Microbial Population Biology, Boston, USA.

2013 Pieczynska, M. D., R. Korona and J. Visser.
"Signature of coevolution between virus killer and its host in yeast strains".
XIVth Congress of the European Society for Evolutionary Biology (ESEB).

2014 Pieczynska, M. D., R. Korona and J. Visser.
"Natural and experimental evolutionary dynamics of *Saccharomyces cerevisiae* killer yeast".
EMBO Conference: Experimental Approaches using Yeast & other Model Systems, Heidelberg, Germany

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Toxins production in killer yeast-the prevalence, mechanism and role in the competition between strains (2010)

Writing of project proposal (4.5 ECTS)

- Role of anti-competitor toxins in the origin and maintenance of diversity in microbial populations (2010)

Post-graduate courses (6.6 ECTS)

- Current improvements in molecular biology; Jagiellonian University, Poland (2011)
- Molecular ecology; Jagiellonian University, Poland (2012)
- Introduction to R for statistical analysis; PE&RC (2013)

Deficiency, refresh, brush-up courses (3 ECTS)

- Introduction to medical biotechnology (2012)
- Population and quantitative genetics (2013)
- Basic statistics (2013)
-

Competence strengthening / skills courses (4.5 ECTS)

- Presentation skills; FNP (Foundation for Polis Science) (2011)
- Scientific writing; FNP (Foundation for Polis Science) (2011)
- Philosophy in life science; Jagiellonian University (2011)
- Spanish language course; Jagiellonian University (2011/2012)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- Symposium: evolution in the laboratory (2013)
- PERC Weekend for the PhD candidates in their last year (2014)
- Annual PE&RC day (2014)
- WEES seminar: adaption and epistasis in laboratory budding yeast (2014)

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)

- Environmental sciences institutes seminars (2010-2012)
- Evolution discussion lunch meetings (2012-2014)
- Experimental evolution discussion group (2012-2014)
- Netherlands Annual Ecology Meeting (NAEM) (2013)
- Monthly WEES seminars (2013-2014)

International symposia, workshops and conferences (9 ECTS)

- 7th International Symbiosis Society Congress: the earth's vast symbiosphere; poster presentation; Krakow, Poland (2012)
- Individuals and groups; oral presentation; Almora, Indie (2012)

- Congress of The European Society for Evolutionary Biology; poster presentation; Lisbon, Portugal (2013)
- Microbial Population Biology GRC conference; poster presentation; Heidelberg, Germany (2014)

Lecturing / supervision of practical's / tutorials (3 ECTS)

- Genetics (2011/2012)
- Microbiology (2011/2012)

Supervision of MSc student

- Prevalence of killer phenotype in feral *Saccharomyces cerevisiae*