Genetic background influences heterogeneity of adaptive response and genome stability during prolonged antifungal drug exposure in *Candida albicans*

Running title: Genetic background influences drug adaptation

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Abstract

The roles of genetic background and within-species diversity in determining the evolutionary potential of a strain to acquire drug resistance are not well understood. To analyze evolution in the presence of fluconazole, a common antifungal drug, we evolved replicates of twenty different clinical isolates of the human fungal pathogen *Candida albicans* from four different clades and with different initial levels of drug resistance in drug for 100 generations. The majority of replicates rapidly increased in fitness in the evolutionary environment, with the degree of improvement inversely correlated with ancestral strain fitness in the drug. Unexpectedly, very few replicates from any strain background acquired *bona fide* drug resistance. Rather, we found prevalent changes in drug tolerance (slow subpopulation growth at high concentrations of drug) and karyotype (changes in chromosome number and/or deviations from diploidy) in a strain-specific manner. Importantly, poor ancestral strain growth in drug was correlated with high levels of heterogeneity among evolved replicates in growth, tolerance, and karyotype, such that the ancestral strain genotype, along with the ancestral fitness level, were associated with the degree of acquired genotypic and phenotypic diversity during adaptation. Thus, the possible responses of a species to a given stress can be diverse and are not necessarily captured by in-depth study of a single strain background. Furthermore, the acquisition of increased fitness in drug is much more frequently associated with a change in tolerance, with or without accompanying karyotypic variation, rather than increased resistance.

Abbreviated abstract

We evolved replicates of twenty different clinical isolates of the human fungal pathogen Candida albicans in drug for 100 generations. Ancestral strain fitness was negatively correlated with fitness improvement in the drug, the heterogeneity of growth among replicates, the level of drug tolerance, and large-scale genome changes (ploidy levels). Improved fitness in drug was much more frequently associated with changes in drug tolerance than with changes in drug resistance.

Impact Statement

The acquisition of drug resistance is a serious threat to global public health. Antimicrobial resistance is inherently an evolutionary phenomenon: in the presence of drug, resistant individuals arise and spread within susceptible populations. The acquisition of resistance is critically understudied in fungi, relative to bacteria, and is usually examined with a single lab or clinical isolate. Unlike bacteria, which frequently acquire plasmid-mediated beneficial genes and alleles from the environment, fungal microbes primarily adapt via vertical transmission (mother to offspring) through a range of both genomic mutations and larger-scale karyotypic changes in ploidy level (the number of chromosome sets) and aneuploidy (the altered copy number of one or several chromosomes). We conducted a large-scale experimental evolution study to probe how different strain backgrounds of *Candida albicans*, a prevalent human fungal pathogen, evolve to fluconazole, the most commonly-prescribed antifungal drug. The results indicate that strain background, mediated in part by the ancestral growth ability in drug, significantly influenced both the mean response and the variability of responses: replicates from strains that grew most slowly when initially exposed to the drug tended to undergo the biggest improvement in growth in drug, but also exhibited more variability in a range of measured phenotypes than replicates from initially fast-growing strain backgrounds. Importantly, very few replicates acquired resistance, defined as the genetically stable ability to grow well at drug concentrations above those used in the evolution conditions. Nevertheless, we found frequent changes in both genome size (DNA content) and antifungal drug tolerance, a newly defined parameter, distinct from resistance, that involves the slow growth of subpopulations of cells in drug concentrations that inhibit the growth of most cells. These results demonstrate that caution should be exercised when generalizing experimental results from a single strain background, and highlight the large range of variability in drug responses within and between populations of microbial populations adapting to drug.

Introduction

The acquisition of drug resistance is an inherently evolutionary process. In principle, a small, finite number of cellular mechanisms can lead to drug resistance, defined as the ability of all cells in a population to grow at drug concentrations above a specific, clinically-established breakpoint, measured as the minimal inhibitory concentration (MIC) of the drug. However, even for well-studied pathogen/drug pairs, the factors that influence the rate at which resistance can be acquired have not been well elucidated.

Drug tolerance in fungi, a property distinct from drug resistance, is the ability of cells to grow slowly at drug concentrations above the minimum inhibitory concentration (MIC; Fridman et al. 2014; Delarze and Sanglard 2015), and correlates well with the size of the subpopulation of cells that can form small colonies on drug as well as the rate at which they do so (Rosenberg et al. 2018).We recently developed the first methods to quantify tolerance using either disk diffusion assays or broth microdilution measurements (Gerstein et al. 2016; Rosenberg et al. 2018) and found that strains that exhibit tolerance wasare more likely to cause clinically persistent infections (i.e., those not resolved by a single course of antifungal therapy) compared to strains with similar MIC levels that are cleared by a single course of the drug (Rosenberg et al. 2018).

The acquisition of drug resistance in eukaryotic pathogens is primarily via vertical transmission of *de novo* mutations, as well as via copy number variations. Karyotypic variation, whether via changes in ploidy (the number of homologous chromosome sets) or aneuploidy (chromosome gain or loss), is frequently observed in laboratory populations subjected to stress (Wellington and Rustchenko 2005; Bouchonville et al. 2009) or passaged through mice (Forche et al. 2009, 2018), as well as in clinical isolates of *Candida albicans, Candida glabrata* and *Saccharomyces cerevisiae* (Ford et al. 2015; Zhu et al. 2016). Karyotype changes appear much more frequently than point mutations (Zhu et al. 2014), and are especially prevalent in strains exposed to azole drug stress (Perepnikhatka et al. 1999; Gerstein et al. 2015; Hickman et al. 2015; Anderson et al. 2017; Hirakawa et al. 2017). The true degree of karyotypic variation among populations is likely to be even higher than what has been observed, as strain isolation methods (i.e., growth on rich medium) may select against karyotypic variants.

A mechanistic link between karyotypic variation and drug resistance has been determined in some pathogenic fungi. Chromosomal aneuploidy for the chromosome carrying *ERG11*, the

target of fluconazole, provides a frequent pathway to drug resistance in both *C. albicans* (chromosome 5 or isochromosome 5L; Selmecki et al. 2006, 2008) and *Cryptococcus neoformans* (chromosome 1, Sionov et al. 2010; Ngamskulrungroj et al. 2012). Additional aneuploidies that increase the ability to grow in fluconazole without a concrete link to overexpression of a particular gene have also been noted in both species (Perepnikhatka et al. 1999; Sionov et al. 2010; Ngamskulrungroj et al. 2012; Anderson et al. 2017; Hirakawa et al. 2017). Intriguingly, certain aneuploidies appear to be beneficial in some strain backgrounds but not in others (Anderson et al. 2017; Hirakawa et al. 2017). Thus, karyotype changes have the potential to provide a rapid, easily reversible, and potentially beneficial response to drug stress. Furthermore, exposure to fluconazole and other azole drugs also can drive karyotypic change by inducing unconventional cell cycle events (Harrison et al. 2014, Altamirano et al. 2017). Hence, fluconazole can both cause, and select for, karyotypic variation within and among populations, and distinguishing between cause and effect is necessary for understanding the mechanistic routes to acquire drug resistance.

The importance of genetic background in the link between genotype and phenotype for *de novo* acquired mutations is increasingly appreciated in both laboratory and natural settings (Chandler et al. 2013; Fournier and Schacherer 2017; Wong 2017, and references within). For example, the phenotype of deleted or repressed genes can vary significantly in different backgrounds (Paaby et al. 2015; Vu et al. 2015) and even the classification of genes as essential for growth under a specific condition can differ between closely related strains (Dowell et al. 2010). The same is true of mutations classified as beneficial in one strain background (including different mutations in the same gene, Peng et al. 2018), which can be neutral or deleterious in other backgrounds (e.g., MacLean et al. 2010; Wang et al. 2013, 2016; Hou et al. 2016). Variation might also arise at different rates due to differences in mutation rate among strain backgrounds (as was recently shown in a *C. albicans* study; Ene et al. 2018). Thus, the mutations available for adaptation, and hence the trajectory of evolution, are thus likely to differ depending on the genetic background of a population. Different genetic backgrounds may well also have different constraints, which should also lead to variation in intra- and inter-population heterogeneity.

Here we set out to explore the interplay between genetic background, resistance, tolerance, and karyotypic variation by following the evolutionary trajectories of replicates from

20 different *C. albicans* strains for 100 generations of evolution in 1µg/ml of fluconazole. We found that the majority of replicates acquired the ability to grow more rapidly in the evolutionary level of drug, with the degree of improvement inversely correlated with ancestral strain fitness in the drug environment. While very few replicates from any background acquired *bona fide* drug resistance, changes in tolerance and karyotype were prevalent, especially in strains with low ancestral growth. Thus, tolerance to drug is an evolvable phenotype, distinct from the acquisition of drug resistance, and it changes much more frequently than resistance. Importantly, ancestral strain fitness was inversely correlated with evolved variation among replicate evolved lines in growth, tolerance, and karyotype, providing a link between strain background and the degree of parallelism among the adaptive trajectories of replicate isolates.

Materials and Methods

Strains

Twenty diploid clinical strains of *Candida albicans* were selected to represent the phylogenetic diversity of the species. The strain set includes at least four strains from each of the four major clades that encapsulate ~70% of the typed *C. albicans* strains and spans nearly the entire known phylogenetic diversity of the species (Odds et al. 2007; Ropars et al. 2018) as well as four commonly-studied laboratory strains (SC5314, FH1, DSY294, T188). For each clade, strains with both heterozygous (*MATa/MATα*) and homozygous (*MATa/MATa, MATα/MATα*) mating loci were chosen. The strains were chosen blind with respect to the initial minimum inhibitory concentration of drug (MIC). Full strain information including clade designation, country of origin, and infection niche were obtained from the original manuscripts (Table 1) and (Tavanti et al. 2005). Mating type genotype was confirmed by PCR with *MATa* and *MATα* specific primers (*MATa* F-TTGAAGCGTGAGAGGCAGGAG, *MATa* R-GTTTGGGTTCCTTCTTTCTCATTC, *MATα* F-TTCGAGTACATTCTGGTCGCG, *MATα* R-TGTAAACATCCTCAATTGTACCCG). All strains were initially streaked onto YPD and grown for 48h at 30°C. A single colony was frozen down in 15% glycerol and stored at −80 °C. Thus, minimal genetic variation should be present in our initial freezer stock, which we refer to throughout as the "ancestral strains".

Evolution Experiment

Strains were evolved in 1 μg/mL fluconazole, the epidemiological cut off value that denotes the upper limit of drug susceptibility (MIC_{50}) in the wild type *C. albicans* population (Delarze and Sanglard 2015). This concentration of drug was equivalent to the ancestral MIC_{50} of three strains, above the MIC_{50} for 11 strains and below the MIC_{50} for one highly resistant strain ($MIC_{50} = 32$) and four additional strains ($MIC_{50} = 4$) (Table 1).

To initiate the evolution experiment we generated twelve independent replicates from each ancestral strain. Cultures were struck from frozen ancestral stocks onto YPD plates (1% yeast extract, 1% peptone, 2% dextrose, 1% agar; the standard lab rich medium) and incubated at 30°C overnight. For each strain, colonies were randomly chosen by spotting each plate twelve times and picking the closest colony to each dot. Colonies were separately inoculated into 1 mL YPD in a 96-well (3 mL) deep culture box and grown shaking overnight at 30 °C.

6

From the overnight cultures, we froze 100 μL from each replicate in duplicate in 50% glycerol as the ancestral replicates. Overnight cultures were diluted 1:1000 into YPD+fluconazole in two steps: first, 10 μL of overnight culture was transferred into 990 μL YPD (a 1:100 dilution), followed by a transfer of 20 uL diluted culture into 180 μL of YPD + 1.11 μg/mL FLC in round bottom microtiter plates. To minimize the likelihood of contamination and keep environmental conditions similar, culture from replicates from one strain was inoculated into row A while culture from replicates from a second strain was inoculated into row H. Plates were sealed with breathe-easy sealing membranes (Sigma Z380058) and incubated static at 30 °C in small sealed rubbermaid containers with wet paper towels inside to minimize evaporation.

After 72 hours, wells were mixed by pipetting and another two-step 1:1000 transfer was conducted into fresh YPD + fluconazole medium. In total 10 transfers were conducted, yielding 100 generations of evolution (9.97 generations between transfers, $\log_2(1000) = 9.97 * 10$ transfers = 99.7 generations). 50μl of the evolved replicate cultures were frozen in duplicate in 50% glycerol after the tenth transfer and maintained at -80 °C.

Resistance and tolerance

The initial susceptibility and tolerance of all strains was tested using broth microdilution liquid assays to measure minimal inhibitory concentration (MIC_{50}) and tolerance as supraMIC growth (SMG), respectively. The liquid assay experiments followed the initial cell dilution regulations from the clinical CLSI M27-A guidelines (http://clsi.org/), except with YPD incubated at 30 °C as the base medium and optical density at A_{600} (OD) instead of a McFarland 0.5 standard to determine the initial density of cells. OD readings were taken at 24 hours after inoculation to calculate MIC_{50} (the point at which optical density was reduced by 50% compared to optical density in YPD without drug).

Four liquid broth microdilution assays were conducted on both the ancestral and evolved replicates, and an additional two assays were conducted on the ancestral replicates. We were not able to assay all drug concentrations in each assay due to capacity (2 time points x 20 strain backgrounds x 12 replicates). A single measurement was taken for each replicate at each concentration of drug measured in a given experiment (see Table S4). The median OD among experiments was determined at each concentration of drug for each replicate. Following

7

guidelines, the MIC_{50} was then calculated as the highest concentration of drug with an OD greater than 50% of the measured OD in YPD (i.e., optical density in medium without drug).

Tolerance was measured from the liquid assay results as in (Rosenberg et al. 2018). The sum of growth (measured as optical density, A_{600}) in the concentrations of drug that were measured beyond MIC_{50} was divided by the number of measured drug concentrations to obtain the average supra-MIC_{50} growth for each ancestral and evolved replicate. This was divided by the measured growth in the lowest drug level (0.0125 uL fluconazole) so that tolerance reflects the fraction of realized growth and is between 0 and 1. Tolerance was assessed at 24, 48 and 72 h. We consider evolved replicates to have increased or decreased in tolerance when their measured tolerance was respectively above or below the range of ancestral tolerance levels for replicates from that strain.

We use "growth" as a proxy measurement for fitness in the evolutionary drug environment, measured as optical density (A_{600}) at both 24 and 72 h. Here, "growth" reflects the ability to convert nutrients from the environment into cellular biomass. OD at 24 h is also consistent with clinical assessment of drug resistance, and correlates with growth rate (Gerstein et al. 2012).

Ploidy variation

Flow cytometry was performed on a BD Biosciences BD LSR II. In all cases, all replicates from the same time point were fixed, stained and measured in parallel. Ancestral replicates were measured twice independently from the freezer stocks maintained at -80 °C. Freezer cultures were thawed, mixed, and 10 μL was added to 500 μL, YPD in deep 96-well boxes, covered with a breathe-easy membrane, and shaken at 200 RPM, 30 °C overnight. After ~ 16 hours of growth, 20 μL of culture was washed in 180 μL of TE, in a round bottom microtiter plate, pelleted, and resuspended in 20 μL TE and fixed by adding 180 μL 95% cold ethanol.

Samples from evolved replicates were fixed in ethanol at the end of the last growth cycle; 50 μL of 72 h culture from t10 was washed in 150 μL TE in a round bottom microtiter plate, pelleted and resuspended in 20 μL TE and 180 μL 95% cold ethanol. Ethanol-fixed cultures were stored at -20 °C for up to 4 weeks. The remainder of the protocol was identical for both time points, following Gerstein et al. 2017. As in Gerstein et al. (2017) we used the cell cycle analysis

in Flow-Jo (Treestar) to determine the mean G1 peak for each replicate; when more than one peak was evident we recorded both the major and minor G1 peaks.

Although we always used the same machine settings, subtle but significant variation is always observed in flow cytometry data. To better compare between t0 and t10 data we performed a day-correction based on the median G1 intensity of the A12 ancestral and evolved replicates, which always measured cleanly as diploids.

Statistical methods

All analysis was conducted in the R Programming Language (R Core Team 2017). To maximize statistical power when testing the influence of mating type, we examined the effect of a heterozygous mating type vs. homozygous mating type, i.e., we combined *MATa/a* and *MATα/α* strains.

MIC_{50} values were log transformed prior to statistical analysis. When parametric tests were used, all assumptions were tested and met. When data transformations were insufficient to meet the test assumptions, nonparametric tests were used. In all cases the specific test is indicated inline.

Results

Replicate lines from 20 different clinical strains of *C. albicans* spanning the phylogenetic diversity of the species (Ropars et al. 2018), and varying in mating type zygosity, geographic origin and site of isolation (Table 1) were evolved in fluconazole (YPD + 1 µg/mL FLC) for 100 generations (20 strains x 12 replicates per strain = 240 replicates total).

Table 1. Strain used in this study. Ancestral minimum inhibitory concentration of drug (MIC_{50}) was measured in YPD at 30 °C.

Strain #	Strain Name	Clade	*MTL* genotype, patient status (if known), site of isolation, country of origin	Aneuploidies	Ancestral MIC	Original Reference
A1	L26	1	a/a, vaginitis, vagina, USA	trisomic chr7	4	Wu 2007
A2	P87	4	a/a, HIV, oral, South Africa	-	1	Wu 2007
A3	GC75	4	α/α, healthy, oral, South Africa	-	0.0125	Wu 2007
A4	P78048	1	α/α, bloodstream, Canada	-	0.5	Wu 2007
A5	P57072	2	α/α, bloodstream, USA	-	4	Wu 2007
A6	P34048	3	a/α, bloodstream, Turkey	-	1	Wu 2007
A7	P37037	1	a/α, healthy, oral, USA	-	1	Wu 2007
A8	P75016	4	a/α, bloodstream, Israel	-	0.5	Wu 2007
A9	P75063	4	a/α, bloodstream, France	-	0.0125	Wu 2007
A10	P76055	2	a/α, bloodstream, USA	-	0.0125	Wu 2007
A11	P78042	3	a/α, bloodstream, USA	trisomic chr4	0.0125	Wu 2007
A12	T101	3	a/a, oropharynx, Canada	-	32	Odds 2007
A13	OKP90	2	a/a, healthy, oral, South Africa	-	0.0125	Lockhard 2002
A14	AM2003.089	2	a/α, oropharynx, UK	-	0.0125	Odds 2007
A15	AM2003.0165	2	α/α, bloodstream, UK	-	0.0125	Odds 2007
A16	AM2003.0069	3	a/α, vagina, UK	-	0.0125	Odds 2007
A17	SC5314	1	a/α, bloodstream, USA	-	0.0125	Fonzi 1993
A18	FH1	3	a/α, marrow transplant, rectal, USA	-	4	Marr 1997
A19	DSY294	11	a/α, HIV, oral, France	-	0.0125	Sanglard 1995, MacCallum 2010
A20	T118	1	a/α, HIV, oral, Canada	-	4	Cowen 2000

## Adaptation is influenced by strain background

The majority of replicates evolved significantly higher growth ability in the evolutionary drug environment (Figure 1). The major exception was replicates from A12, the strain with the highest ancestral MIC_{50}, which evolved significantly lower 24 h growth and no change in average growth at 72 h. Four strains with initial MIC ≤ 1 (A2, A9, A10 and A14) also did not acquire improved average growth at 24 h, though on average all had significantly increased growth at 72 h (significance determined as appropriate by parametric or nonparametric t-tests, methods and detailed results in Table S1).
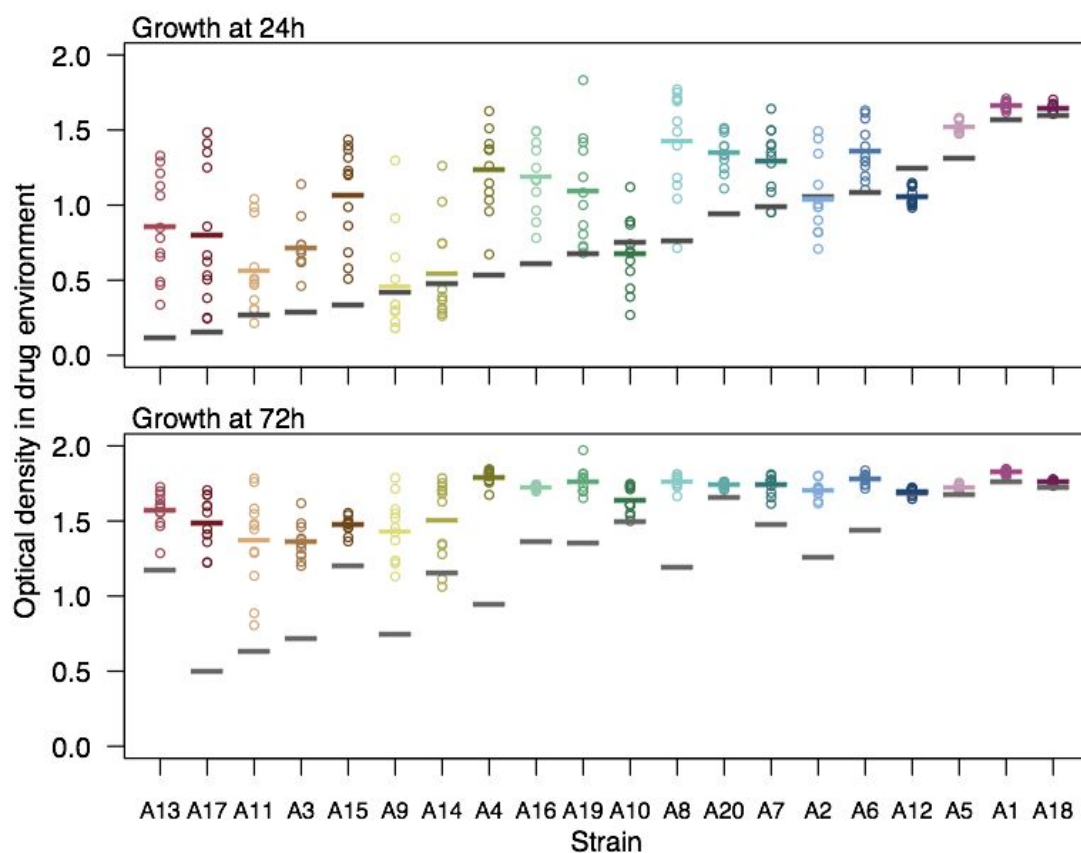
Figure 1. The majority of evolved replicates improved growth in the evolutionary environment after ~100 generations of evolution. Fitness was measured as optical density in YPD + 1 µg/mL fluconazole, the evolutionary environment after 24 h (top) and 72 h (bottom). Strains are ordered by ancestral growth in the evolutionary environment at 24 h. Ancestral growth ability (the median growth among 12 ancestral replicates) is indicated for each strain by a grey bar. Each coloured point represents one of twelve evolved replicates while the coloured bars indicate median evolved growth for visual comparison to ancestral growth.

Ancestral strain growth strongly influenced the mean degree of growth improvement among replicates, while neither mating type nor clade had a significant effect (Figure 2, ANOVA tests; 24 h—ancestral growth: $F_{1, 13} = 7.99$, $p = 0.014$, clade: $F_{4, 13} = 0.64$, $p = 0.64$, *MAT* zygosity: $F_{1, 13} = 0.27$, $p = 0.61$; 72 h—ancestral growth: $F_{1, 13} = 158.73$, $p < 0.0001$, clade: $F_{4, 13} = 1.97$, $p = 0.16$, MAT zygosity: $F_{1, 13} = 0.25$, $p = 0.63$). The change in growth (difference between evolved and ancestral replicates) and the ancestral growth were significantly correlated at both time points (Pearson's correlation; 24 h: $t18 = -3.01$, $p = 0.007$, cor = -0.58. 72 h : $t_{18} = -11.63$, $p < 0.00001$, cor = -0.94). The influence of strain background is evident in those strains that deviated considerably from the correlation line of best fit, particularly at 24 h (Figure 2).

The variance among evolved replicates from each strain reflects stochasticity in the evolutionary process. Variance in evolved growth and the degree of growth improvement were inversely correlated: evolved replicates that were least fit initially and that increased in growth the most also had the most variability among replicates from the same strain background (Spearman's rank correlation test; 24 h: S = 694, $p = 0.035$, rho = 0.48. 72 h: S = 476, $p = 0.003$, rho = 0.64). Overall, strain background influenced both the degree of growth improvement and the growth variability among replicates, both mediated in part by the ancestral growth ability of the strain in the evolutionary environment.
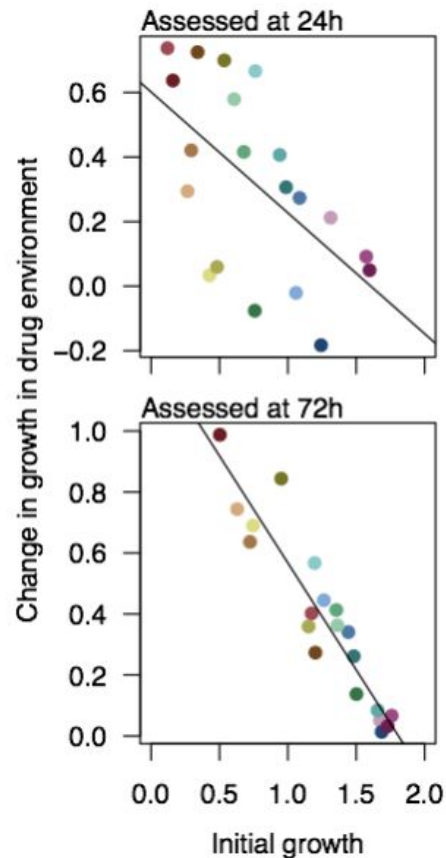
Figure 2. Significant negative correlation between ancestral growth and change in growth. Growth was measured as optical density in YPD+1 µg/mL fluconazole after 24 h (top) and 72 h (bottom). The colour matches Figure 1, based on ancestral growth (low ancestral growth strains are coloured red, with colours proceeding as the colours of the rainbow to the highest initial 24 h growth strain in violet).

Minimal tradeoff to permissive environment

A tradeoff is often seen between the cost and benefit of mutations that enable microbes to grow well in antimicrobial drugs, although several drug classes and pathogen species do not impart a major cost (Melnyk et al. 2015, and references within). A similar pattern of species/drug specificity on tradeoffs appears for mutations that provide an advantage to fungi exposed to antifungal drugs. For example, the ability to grow in nystatin incurs a significant cost in *S. cerevisiae* (Gerstein et al. 2012), yet mutations that confer an advantage to high concentrations of fluconazole in *C. albicans* incur no appreciable cost (Anderson et al. 2003; Selmecki et al. 2009). The degree to which there is a cost-benefit tradeoff for growth in lower concentrations of fluconazole is not known.

Strain background had a significant impact on the tradeoff between growth in 1 µg/ml fluconazole and growth in the absence of the drug: 80% of evolved replicates grew more slowly in the drug-free environment at 24 h, with an average growth reduction of 21% compared to the ancestral replicates (Figure 3). Exceptions were strain A5, where the majority of evolved replicates showed a significant growth improvement (6% on average), and strains A8, A10 and A11, where evolved replicates had no significant change on average (Table S2). Strain background influenced growth at 72 h (Table S2; average growth improvement of 1%: A1, A4, A5, A10, A11, A12, A20; average growth reduction of 5%: A3, A13, A14, A15, A17). Overall, the majority of the evolved replicates exhibited slower initial growth in a drug-free environment, yet reached the same or slightly higher growth by 72 h. The primary trade-off for the drug-free environment was thus in the initial phase of growth, suggesting an increased amount of time spent prior to rapid cell division.

Mean growth in the drug and drug-free environments at 24 h was significantly correlated among strain backgrounds (Spearman's rank correlation test; S = 396, *p* = 0.0008, rho = 0.702, Figure S1; there was not enough variation in the drug free environment at 72 h for correlative purposes). A significant positive relationship was also recovered when comparing among evolved replicates of the same strain background for 12 of 16 strain backgrounds with variable replicates (the four backgrounds with the highest ancestral growth had extremely low variation in in both the presence or absence of fluconazole; Figure S2). Thus, the overall pattern among strain backgrounds and different replicates of the same strain was similar: strains (and replicates) that evolved higher growth in low drug also had an advantage in the no drug environment.

13

Hence, mutations acquired in response to a low concentration of fluconazole had an initial cost at 24 h in the no-drug environment, though this was mitigated when growth was allowed to proceed for 72 h.
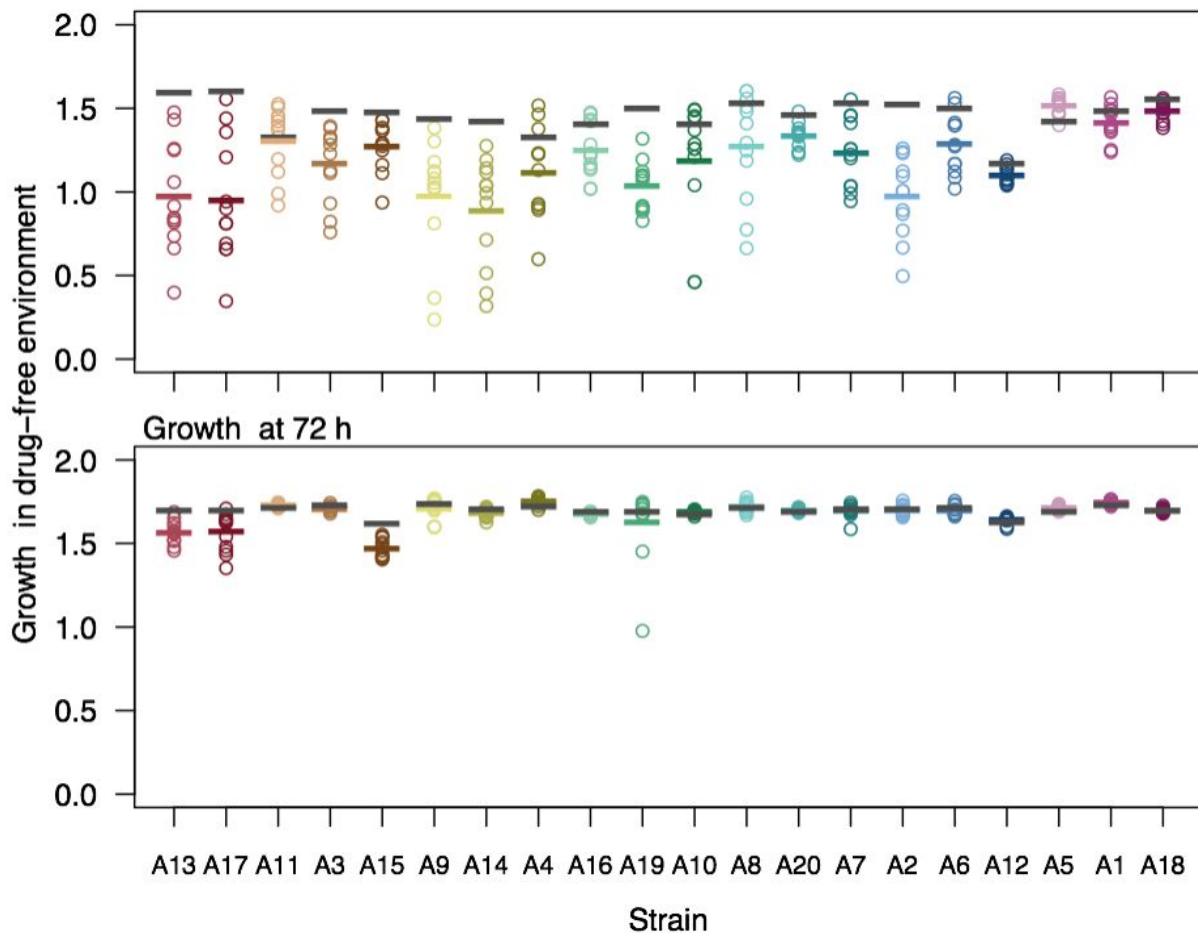

Figure 3. Growth in a drug-free environment. Growth was measured as optical density in YPD, the standard lab rich medium, after 24 h (top) and 72 h (bottom). Strains are ordered by ancestral growth ability in the low drug evolutionary environment. Ancestral growth in YPD is indicated by a grey bar. Each coloured bar represents the median of twelve replicates evolved from each ancestral strain.

Drug resistance increases were rare while changes in drug tolerance were common

Drug susceptibility/resistance is a concentration-dependent property of individual strains measured as MIC_{50}. Only 18 of the 240 replicates increased in MIC_{50} beyond the evolutionary level, hailing from seven of the 20 strain backgrounds (Figure 4), with only slight increases in MIC_{50} in the majority of cases (to 4 μg/mL; five replicates from strain A3, four replicates from A8, and one replicate from A4 and A7). Clinical resistance ($MIC_{50} \geq 8$ μg/mL at 24 h, (Fothergill et al. 2014) was acquired in replicates from only three backgrounds: six from strain A5 (initial MIC_{50} of 4 μg/mL) and one each from strains A17 and A19 (initial MIC_{50} of 0.0125 μg/mL).

14

Interestingly, MIC_{50} decreased to 1μg/mL in some replicates from the five strains with initial MIC_{50} >1 μg/mL (two replicates from A5, eight from A12, all 12 from A20, Figure 4). This suggests a fitness cost of initially high MIC_{50} during prolonged growth in low concentrations of drug in some, but not all, strain backgrounds.
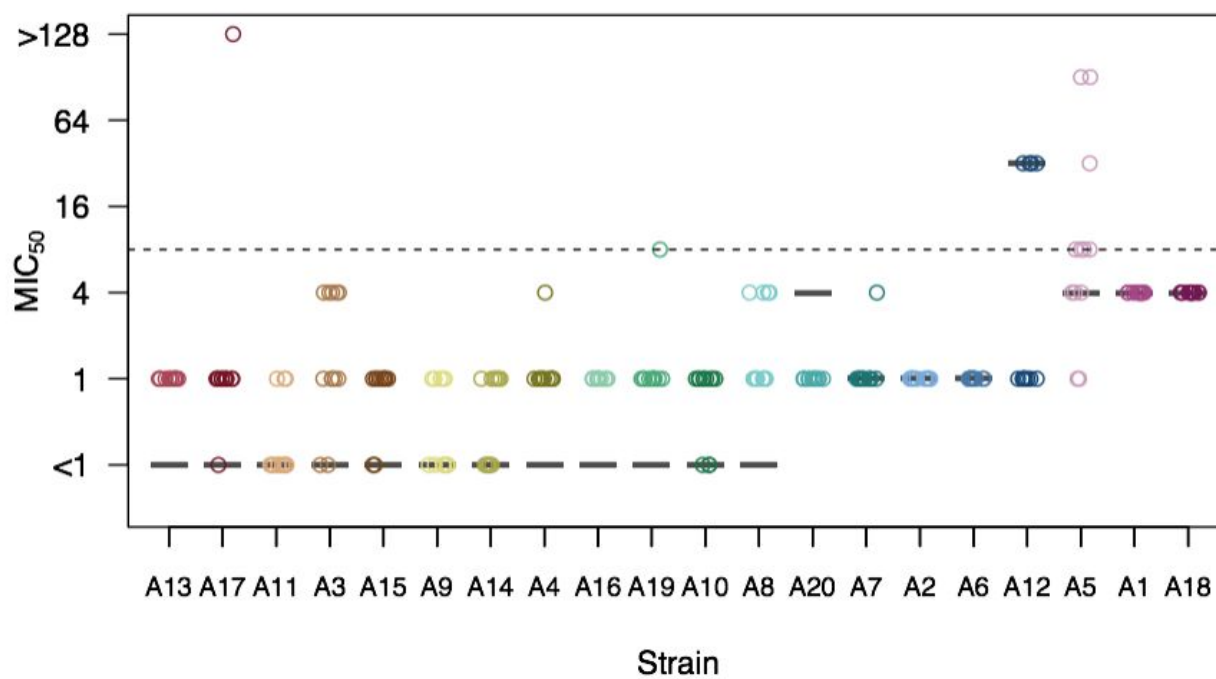
Figure 4. The majority of evolved replicates did not acquire resistance. Drug resistance was measured through broth microdilution assays. The clinical cutoff for resistance at 8 μg/mL fluconazole is indicated by the dashed line. Strains are arranged on the x-axis (and coloured) by initial growth in the evolutionary environment. Each point represents an individually-evolved replicate line.

Increases and decreases in tolerance were prevalent among evolved replicates (Figure 5, Figure S3), unlike the infrequent evolution of increased resistance. Approximately one third of the evolved replicates increased tolerance (24h: 72 replicates from 13 different strain backgrounds; 72h: 73 replicates from 13 strain backgrounds), while a similar number of replicates decreased tolerance at both time points (24 h: 79 replicates from 17 strain backgrounds; 72 h: 91 replicates from 17 strain backgrounds). The magnitude of tolerance change increased sharply between 24 and 48 h of drug exposure (Figure S3), consistent with a previous examination (Rosenberg et al. 2018). To match the timing of our transfers and growth measurements, we primarily considered tolerance at 72 h as a measure of tolerance in evolved replicates (tolerance at 48 h and 72 h are very similar, Figure S3). The number of replicates that increased or decreased in tolerance varied considerably among strain backgrounds (Figure 5, Figure S4).

15
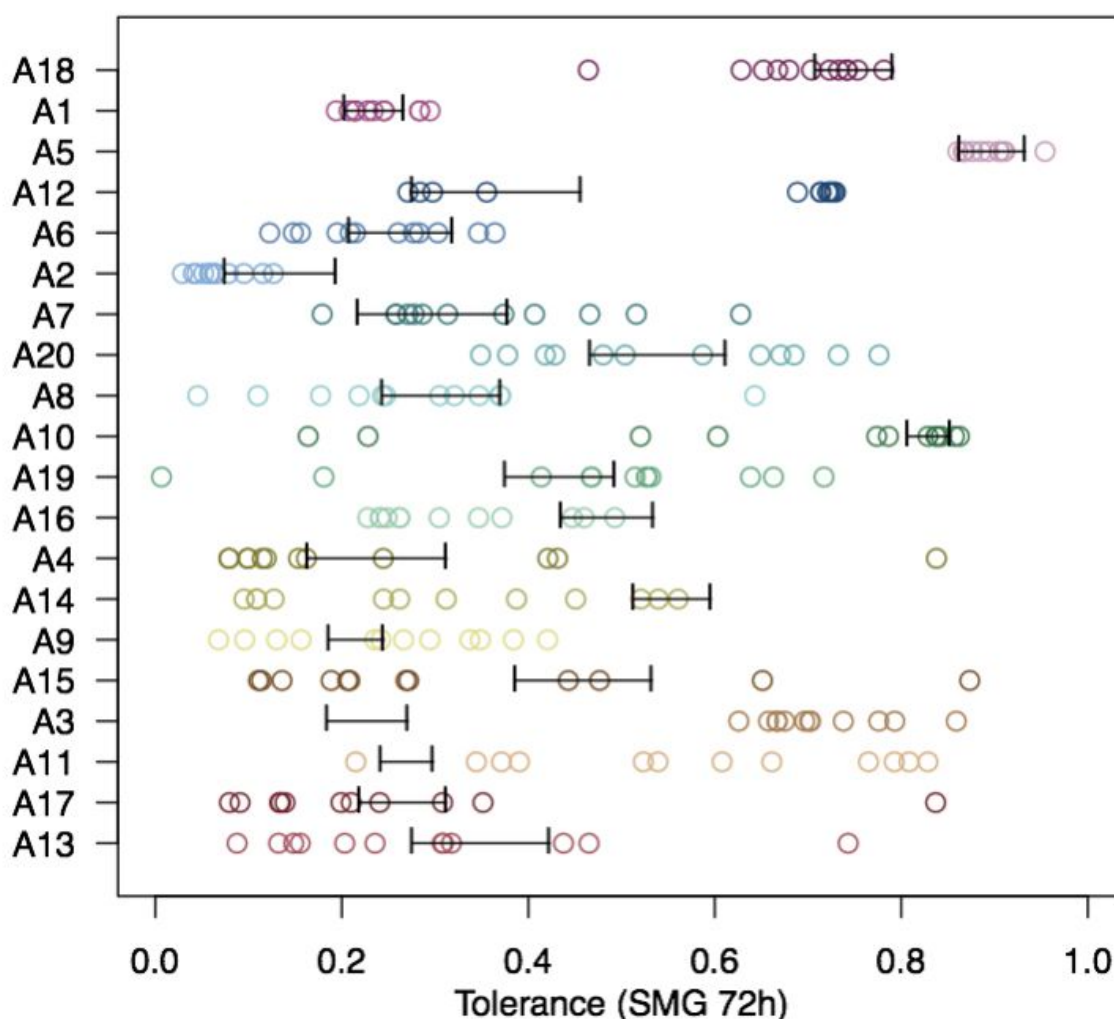
Figure 5. Many evolved replicates changed in tolerance. Tolerance was measured as the growth observed in supra-MIC levels of fluconazole normalized to the growth in a very low level of drug after 72 h. Strains are arranged in order of increasing growth in the evolutionary environment at 24 h (bottom-top here corresponds to left-right in Figure 1). The black lines indicate the range of tolerance values measured among ancestral replicates.

Notably, in some strain backgrounds nearly all replicates changed in the same direction, while in others the replicates were split between those that increased and those that decreased evolved tolerance. Change in tolerance was not significantly correlated with initial growth, clade, or mating type (*MAT* locus) status in the evolutionary environment (linear mixed-effects model with change in tolerance as the response variable and initial growth, zygosity and clade as predictor variables and strain as a random effect; initial growth: $F_{1,217} = 0.51$, $p = 0.47$, clade: $F_{4,14} = 1.79$, $p = 0.19$, MAT zygosity: $F_{1,14} = 2.53$, $p = 0.13$). Variation in evolved growth and the variance in tolerance were positively correlated (Spearman's rank correlation: growth measured

16

at 24 h: S = 588, p-value = 0.012, rho = 0.56; this relationship was not statistically significant for variability in growth at 72 h though rho was still relatively high: S = 762, p = 0.06, rho = 0.43). Thus, genetic background, mediated in part by ancestral growth, influenced the evolved variation among replicates in the evolution of tolerance as well as resistance.
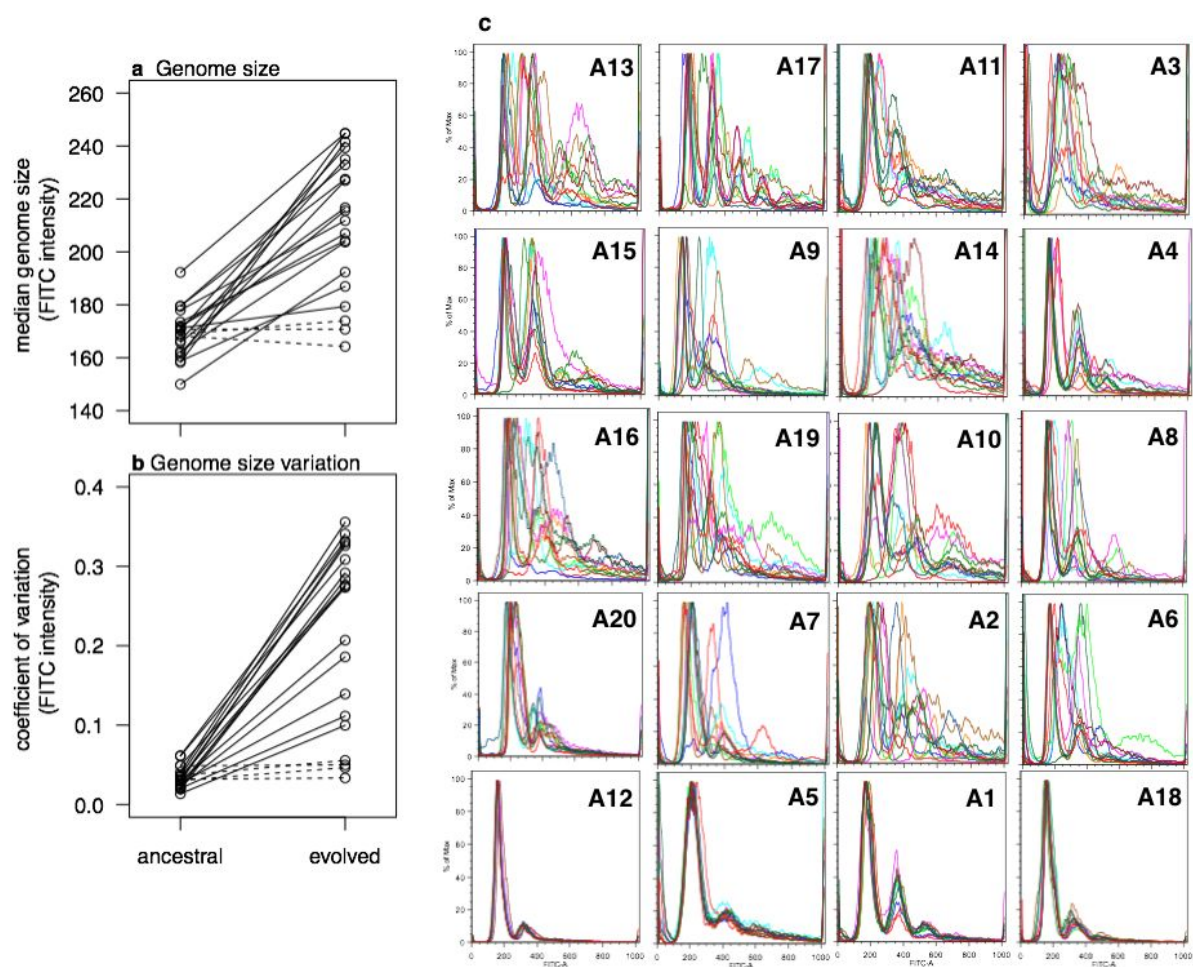
Genome size changes are pervasive

Exposure to fluconazole is known to induce the formation of tetraploid, and subsequently aneuploid, cells (Harrison et al. 2014) and specific aneuploidies can be selectively advantageous (Selmecki et al. 2006; Yang et al. 2013; Anderson et al. 2017). Here, evolved replicates underwent a significant increase in median genome size across the majority of strain backgrounds (Figure 6A, Table 2). The three exceptions were A12, the resistant strain; and A4 and A9, initial MIC_{50} values < 1 µg/mL. The coefficient of variance for ploidy level (a measure of variability among replicates) also increased for the majority of strains (Figure 6B). Thus, genome size variation among replicates increased over time in drug, as expected if evolved replicates acquired different final numbers of chromosomes.

Importantly, the least ploidy variation was seen in replicates evolved from the four parental strain backgrounds that had the highest initial growth levels in low drug and MIC_{50} above the drug concentration used (1 µg/mL fluconazole) (Figure 6C, bottom four panels). When these four strains were removed from the analysis, median evolved genome size was neither correlated with ancestral growth in fluconazole (Pearson's correlation, 24h: t14 = -0.34, p = 0.74; 72 h: t14 = -0.32, p = 0.75), nor with the change in low drug growth (Spearman's rank correlation, 24 h: S = 541.19, p = 0.45; 72 h: S = 953.4, p = 0.12), indicating that a different factor influences propensity to acquire karyotypic changes among strain backgrounds.

Table 2. Welch two sample t-tests to compare ancestral and evolved genome size.

Strain	Statistics
A1	$t_{20.6}$ = 11.70, p < 0.0001
A2	$t_{11.2}$ = 5.34, p = 0.002
A3	$t_{11.1}$ = 5.69, p = 0.0001
A4	$t_{11.4}$ = 1.91, p = 0.08
A5	$t_{20.14}$ = 18.07, p < 0.0001
A6	$t_{11.1}$ = 3.82, p = 0.003
A7	$t_{11.1}$ = 2.80, p = 0.017
A8	$t_{11.1}$ = 2.80, p = 0.017
A9	$t_{11.2}$ = 0.88, p = 0.40
A10	$t_{11.4}$ = 3.31, p = 0.007
A11	$t_{11.5}$ = 5.24, p = 0.0002
A12	$t_{18.8}$ = 0.71, p = 0.49
A13	$t_{11.1}$ = 5.46, p = 0.0002
A14	$t_{11.1}$ = 4.10, p = 0.002
A15	$t_{11.1}$ = 2.79, p = 0.018
A16	$t_{11.3}$ = 4.17, p = 0.0015
A17	$t_{11.1}$ = 2.94, p = 0.013
A18	$t_{18.4}$ = 2.98, p = 0.008
A19	$t_{11.4}$ = 2.93, p = 0.013
A20	$t_{11.5}$ = 5.91, p < 0.0001

Figure 6. Genome size and variation in genome size increased after evolution to low fluconazole. a) Median genome size, and b) coefficient of variation (CV; i.e., variability among replicates) in each strain background. A dashed line indicates a non-significant change between ancestral and evolved replicates. c) Flow cytometry traces of each replicate evolved line, arranged by ancestral strain growth in low fluconazole from lowest (A13) to highest (A18).

These median numbers, however, obscure the tremendous variation in ploidy level and evolved growth observed among independently evolved replicates. Comparison of evolved genome size and evolved growth for replicates from the 16 strain backgrounds that evolved variable genome size revealed that evolved growth at 24 h and evolved genome size correlated significantly in five strains: negatively in A6, A8, A10 and A16 (replicates that increased in genome size tended to have less growth at 24 h), and positively in A13 (replicates that increased in genome size tended to have more growth at 24 h; Figure 7, Table S3). Evolved growth in strain A13 at 72 h remained positively correlated with genome size. By contrast, two different strains (A2 and A4) had weak, but significant negative correlations between growth at 72 and evolved genome size (Figure S4, Table S3). Across strain backgrounds, no clear association between alterations in genome size and increases in MIC_{50} (Figures 7, filled points), or evolved changes in tolerance

18

(Figure S5) were evident. Importantly, once again, the variance in evolved genome size correlated with ancestral growth (Pearson's correlation, $t_{18} = -3.22$, $p = 0.0047$, cor = -0.60) and the variance in evolved growth (Pearson's correlation, $t_{18} = 5.65$, $p = 0.00002$, cor = 0.80). Hence, there was no direct link between evolved genome size and evolved growth in most strain backgrounds, yet the variability among replicates is consistently larger in some strain backgrounds than others, mediated in part by ancestral strain growth in the evolutionary environment.
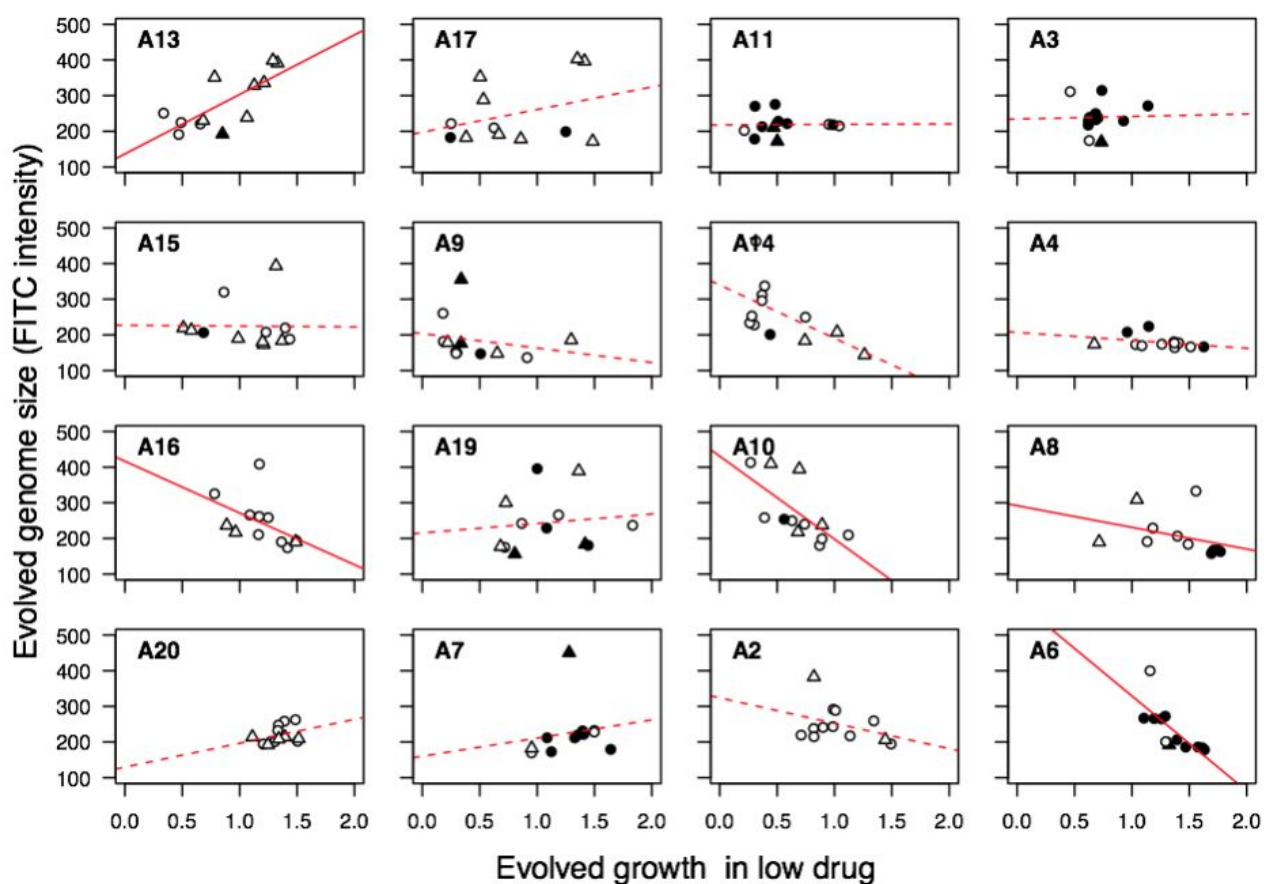
Figure 7. Evolved growth and evolved genome size are not consistently correlated. Growth was measured as optical density after 24 hours in low fluconazole. Evolved genome size indicates the most prominent G1 peak; when multiple G1 peaks were present this is indicated with a triangle symbol. Solid lines indicate a significant correlation. Each point represents an independently evolved replicate line. Filled-points are those lines that increased in MIC beyond the level of drug stress (1 µg/mL fluconazole) they were evolved in. Strains are arranged from the lowest initial growth (top left) to highest (bottom right). Replicates from the four strains that had ancestrally high MIC levels above the evolutionary environment did not evolve variation in genome size and are not presented here.

Discussion

The acquisition of *bona fide* drug resistance by microbial pathogens is inherently an evolutionary process. Here, we evolved 240 replicates from 20 *C. albicans* strains to 1 µg/ml fluconazole, a drug concentration above the ancestral MIC_{50} for the majority of strains. A short period of laboratory evolution (100 generations) was chosen to capture the first one or several beneficial mutations in each replicate population. The majority of replicates rapidly increased in growth ability in the evolutionary environment in a manner inversely correlated with ancestral strain growth in the drug. This pattern of diminishing-returns for fitness is similar to previous experimental evolution studies in bacteriophage (eight genotypes, Rokyta et al. 2009) and *E. coli* (five strains, Moore and Woods 2006). It is also consistent with a suite of other studies: a negative correlation between ancestral fitness and rate of adaptation in strains that differ by many mutations (Jerison et al. 2017) and in strains differ by only one or a few beneficial (Kryazhimskiy et al. 2014; Wünsche et al. 2017) or deleterious mutations (Perfeito et al. 2014); the effect size of a beneficial mutation tends to be smaller in a background that already contains a different beneficial mutation (Chou et al. 2011; Khan et al. 2011; Ono et al. 2017); the effect size of beneficial mutations transferred onto a diversity of strain backgrounds is negatively correlated with ancestral fitness (Wang et al. 2016); and compensatory adaptation occurs more rapidly for severely deleterious mutations compared to weakly deleterious mutations (Moore et al. 2000; Sanjuán et al. 2005; Barrick et al. 2010). Through simulation analyses, Couce and Tenaillon (2015) found a robust match between these observed patterns and Fisher's abstract geometric model (Fisher 1930; Tenaillon 2014), providing a theoretical underpinning for the negative relationship between the distance from the fitness optimum (ancestral fitness) and the number and/or effect size of beneficial mutations (rate of adaptation). We thus predict to uncover a negative correlation between ancestral growth ability and the effect size of the mutations that underpin the observed fitness improvements, a hypothesis we will test in f\uture work.

Of note, in our study the correlation between ancestral growth and change in growth was stronger when growth was measured at 72 hours (adjusted r-squared from the log-log regression = 0.96) compared to 24 hours, the time when MIC is clinically determined (adjusted r-squared = 0.72). Thus, although the pattern of a declining rate of adaptability with increased ancestral growth holds across species (e.g., (Couce and Tenaillon 2015), it is worth considering whether some aspects of the strain background influence deviations from this pattern, particularly during

20

adaptation to a fungistatic drug such as fluconazole, which inhibits cell growth. Six strains had a notably lower growth improvement than expected at 24 h (Figure 2A). Nothing that we know about these strains distinguishes them—they are from different phylogenetic clades, include both homozygous and heterozygous mating loci, and were isolated from diverse geographic locations and clinical niches. As we conduct additional experiments with these strains it will be interesting to see whether the same strains repeatedly behave as outliers, and to further probe how the role of tolerance vs resistance affects growth measurements and the interpretations of the role of ancestral fitness.

Very few (<5%) of replicates with initial MIC_{50} at or below 1 µg/mL fluconazole increased their MIC_{50} to > 1 µg/mL, in contrast to the rapid growth improvements observed in the evolutionary environment and widespread changes in tolerance. Thus, there was only a small window of MIC_{50} improvement that did not extend beyond the concentration of drug in which the replicates were evolved. The influence of subinhibitory drug concentrations on MIC_{50} remains under-studied in fungi. In *Sclerotinia sclerotiorum,* a plant pathogenic fungus, no consistent relationship was found between increased resistance after repeated exposure to sublethal concentrations of different antifungal drugs. In three different drugs, approximately half of the strains increased in MIC_{50}, while in two additional drugs almost no strains acquired increased MIC_{50} (Amaradasa and Everhart 2016)**.** By contrast, many studies in bacteria have found that exposure to subinhibitory concentrations of antibiotics can select for *de novo* mutations that confer resistance (Wu et al. 1999; Henderson-Begg et al. 2006; Gullberg et al. 2011; Chow et al. 2015; Torres-Barceló et al. 2015; Wistrand-Yuen et al. 2018) or augment existing resistance mutations (e.g., (Gullberg et al. 2011; Liu et al. 2011; Mira et al. 2015)(McVicker et al. 2014; Lundström et al. 2016)(Gullberg et al. 2011; Liu et al. 2011; Mira et al. 2015); (McVicker et al. 2014; Lundström et al. 2016). This highlights the important influence of different drugs as well as the potential differences between different organisms on the breadth and molecular influence of drug resistance mutations.

Strikingly, one-third of evolved replicates with ancestral MIC_{50} above 1µg/mL fluconazole (22 of 60) evolved a reduced MIC_{50} (i.e., higher of susceptibility to fluconazole). The four strains that initially had an MIC_{50} of 4 µg/mL exhibited patterns consistent with an effect of strain background on the propensity for MIC_{50} reduction: all replicates from two backgrounds (A1, A18) maintained an MIC_{50} of 4 µg/mL, while the MIC_{50} decreased to 1 µg/mL

in all 12 replicates from strain A20 (fisher-exact test, p < 0.00001). Replicates from the fourth strain (A5) were extremely variable, as two replicates decreased to MIC_{50} 1 µg/mL, while seven increased to > 4 µg/mL, and the remainder did not change. Replicates from a fifth strain, A12, which was initially clinically resistant at MIC_{50} 32 µg/mL, also evolved a lower MIC_{50}. That few ancestrally low-MIC_{50} replicates increased in MIC_{50}, while many ancestrally-high MIC_{50} replicates decreased in MIC_{50}, suggests that a cost-benefit trade-off exists between alleles that provide high fitness in relatively low drug concentration and those that provide high fitness in high drug, in at least some strain backgrounds.

Drug tolerance, the ability of a subpopulation to grow slowly at high drug concentration, changed more frequently than resistance following adaptation to drug. Over half of the replicates evolved an altered tolerance level, with both increases and decreases in tolerance detected. This implies that the ability of a subpopulation to grow in drug concentrations beyond the MIC_{50} evolves at a higher frequency than does the acquisition of *bona fide* resistance. There were significant differences among strains in the propensity for tolerance levels to change yet a lack of correlation of evolved tolerance with ancestral growth levels, MIC_{50}, or known strain characteristics (i.e., *MAT* genotype or clade). Since tolerance is affected by many genetic pathways and environmental conditions and can likely be influenced by a large range of genetic targets (Gerstein et al. 2016; Rosenberg et al. 2017), the evolution of tolerance is likely to be highly dependent upon the genetic background of a given strain, the environmental conditions, and the specific mutations that are selected in each replicate population.

The appearance of karyotypic variability among evolved replicates was strain-dependent and influenced by initial fitness: the four strains with ancestral MIC_{50} > 1 µg/mL did not exhibit any change in genome size variability after 100 generations. By contrast, karyotypic variability increased significantly for all other strain backgrounds. Notably, the degree of karyotype variability among replicates from a given strain correlated significantly with the degree of variability in both evolved growth and evolved tolerance. This was driven, in part, by the much lower variability in karyotypes and other phenotypes for strains with MIC_{50} above 1µg/ml and is presumably because such cells experience far less stress in the evolutionary environment. This highlights the importance of the relative relationship between drug concentration and initial strain fitness: the same drug concentration can be 'low stress' in one strain yet likely yields a 'high stress' response in a different strain. The low evolved ploidy variability observed for

strains with initial MIC above the drug evolutionary environment (1μg/ml fluconazole) is indicative of this difference.

Importantly, there was a broad range of evolved variability among strains with $MIC_{50} <$ 1μg/ml fluconazole. Thus, some strain backgrounds were more likely to yield evolved populations with variable phenotypes than others, regardless of the phenotype being measured (i.e., growth ability, tolerance, genome size), yet the actual phenotype parameter values were not necessarily correlated with each other or even in the same direction. This implies that the ability to adapt to a wide range of different environmental stresses is likely to be very dependent upon the ancestral genetic background. Given the broad range of responsiveness in the different strains exposed to similar levels of stress ($MIC_{50} <$ 1μg/ml), we suggest that many small genetic differences and the molecular pathways that underpin them are likely to be involved in the process of tolerating drug stress.

Previous studies found a higher level of fluconazole (10 μg/ml) both promotes the formation of tetraploid and aneuploid cells and also selects for specific aneuploidies (Selmecki et al. 2006; Harrison et al. 2014). Here, a 10-fold lower concentration of fluconazole also yielded widespread aneuploidy, presumably via similar mechanisms. The fact that very few replicates acquired *bona fide* drug resistance, while nearly all improved in growth in the evolutionary environment and many acquired altered tolerance levels, indicates that the responses involved in growth and tolerance to 1μg/mL fluconazole are distinct from those aneuploidies selected at 10 μg/ml that yielded much higher MIC_{50} levels. Furthermore, it suggests that many more genes and genetic pathways likely influence overall growth and tolerance than the small number of genes that directly affect *bona fide* fluconazole resistance. In other words, the target size for genes affecting fitness at low drug and drug tolerance is likely much larger than that for *bona fide* resistance.

Conclusion

Experimental evolution studies provide an extremely useful framework to isolate important factors that influence adaptation. Here, we found that genetic background had a significant influence on the rate and variability of adaptation, mediated in part through ancestral fitness relative to the selective conditions used. Fitness improvements were largely restricted to the fluconazole drug concentration used in the evolution experiment, and were indicative of a

23

tradeoff between fitness at low and high concentrations of drug. Changes in ploidy were prevalent among replicates from strains that had ancestral MIC below the drug concentration used in the evolutionary environment, yet was largely absent from ancestral strains with MIC levels above it. This implies that drug stress is context-dependent: the level of stress conferred by exposure to a high concentration of drug to one strain may be relatively benign to another. Importantly, variability among replicates was correlated for multiple evolved phenotypes, and strains with similar ancestral fitness did not always yield the same degree of variability. Thus, strain background is an important factor that needs to be taken into consideration explicitly when making generalizations about adaptation and stress responses.

24

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Author Contributions

All authors designed the study and wrote the manuscript. ACG conducted the research and analyses.

Data Accessibility

All data and the R code required to run the analyses and create the visualizations will be deposited in the Dryad data repository prior to publication.

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29

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Supplementary Tables and Figures

Table S1. The majority of strains evolved an increased growth ability in the evolutionary environment. The test column indicates the test that was run: a t-test when both ancestral and evolved replicate groups were normally-distributed with equal variance, a t-test with welch approximation for degrees of freedom when variances were unequal, or the Wilcoxon Rank Sum test when the data from at least one group was not normally distributed. Equal variance was assessed with an F test, normality with the Shapiro-Wilk test (assumption test results not shown).

Line	Growth ability at 24 h					Growth ability at 72 h				
	test	t-value	df	p	evol-anc	test	t-value	df	p	evol-anc
A1	t-test	-6.85	22	<0.0001	0.09	t-test	-12.49	22	<0.0001	0.07
A2	wilcox	89	-	0.35	-0.02	wilcox	0	-	<0.0001	0.45
A3	wilcox	0	-	<0.0001	0.42	t-test	-12.96	22	<0.0001	0.64
A4	welch	-9.02	11.47	<0.0001	0.7	wilcox	0	-	<0.0001	0.84
A5	welch	-5.58	13.31	0.0001	0.21	t-test	-8.49	22	<0.0001	0.05
A6	t-test	-4.29	22	0.0003	0.27	wilcox	0	-	<0.0001	0.34
A7	t-test	-4.17	22	0.0004	0.31	t-test	-7.95	22	<0.0001	0.26
A8	welch	-6.67	11.79	<0.0001	0.67	welch	-19.22	15.01	<0.0001	0.57
A9	wilcox	96	-	0.18	0.03	welch	-11.17	12.63	<0.0001	0.69
A10	welch	1.03	13.14	0.32	-0.08	wilcox	7	-	<0.0001	0.14
A11	welch	-3.58	11.5	0.0041	0.29	welch	-8.09	12.45	<0.0001	0.74
A12	t-test	7.26	22	<0.0001	-0.18	wilcox	36	-	0.0387	0.01
A13	welch	-7.45	11.16	<0.0001	0.74	welch	-10.56	15.23	<0.0001	0.4
A14	wilcox	94	-	0.22	0.06	wilcox	21	-	0.0023	0.36
A15	welch	-7.35	12.41	<0.0001	0.72	t-test	-9.91	22	<0.0001	0.27
A16	welch	-8	14.74	<0.0001	0.58	welch	-21.39	12.27	<0.0001	0.36
A17	welch	-4.77	11.07	0.0006	0.64	welch	-18.68	14.55	<0.0001	0.99
A18	t-test	-4.92	22	0.0001	0.05	t-test	-6.34	22	<0.0001	0.03
A19	welch	-3.9	11.83	0.0022	0.42	t-test	-12.61	22	<0.0001	0.41
A20	t-test	-9.59	22	<0.0001	0.41	t-test	-11.5	22	<0.0001	0.08

Table S2. Replicates from the majority of strain backgrounds evolved a reduced growth ability at 24 hours in a drug-free environment in the evolutionary environments. The test column indicates the test that was run: a t-test when both ancestral and evolved replicate groups were normally-distributed with equal variance, a t-test with welch approximation for degrees of freedom when variances were unequal, or the Wilcoxon Rank Sum test when the data from at least one group was not normally distributed. Equal variance was assessed with an F test, normality with the Shapiro-Wilk test (assumption test results not shown).

Line	Growth ability at 24 h					Growth ability at 72 h				
	test	t-value	df	p	evol-anc	test	t-value	df	p	evol-anc
A1	welch	2.38	14.79	0.0311	-0.08	t-test	-3.61	22	0.0016	0.02
A2	welch	7.54	12.91	<0.0001	-0.55	t-test	0.93	22	0.36	-0.01
A3	welch	4.75	12.75	0.0004	-0.31	t-test	2.98	22	0.0069	-0.02
A4	welch	2.56	12.68	0.0241	-0.21	t-test	-3.63	22	0.0015	0.03
A5	welch	-2.78	16.69	0.0129	0.09	t-test	-3.59	22	0.0016	0.02
A6	welch	4.06	12.35	0.0015	-0.21	welch	1.11	15.03	0.28	-0.01
A7	wilcox	134	-	0.0001	-0.3	wilcox	76.5	-	0.82	-0.01
A8	wilcox	104	-	0.068	-0.25	t-test	-0.85	22	0.4	0.01
A9	wilcox	142	-	<0.0001	-0.46	wilcox	77.5	-	0.77	-0.03
A10	wilcox	99	-	0.13	-0.22	t-test	-2.47	22	0.0219	0.01
A11	welch	0.35	12.66	0.73	-0.02	t-test	-3.03	22	0.0062	0.02
A12	t-test	2.95	22	0.0074	-0.07	wilcox	33	-	0.0242	0.01
A13	welch	6.6	11.07	<0.0001	-0.62	welch	6.61	11.63	<0.0001	-0.13
A14	welch	5.59	11.55	0.0001	-0.53	welch	3.12	14.1	0.0075	-0.03
A15	welch	4.73	11.69	0.0005	-0.2	welch	9.55	11.41	<0.0001	-0.15
A16	welch	2.98	13.84	0.0101	-0.15	t-test	2.05	22	0.053	-0.01
A17	welch	6.19	11.03	0.0001	-0.66	welch	3.86	11.27	0.0025	-0.13
A18	welch	4.11	12.17	0.0014	-0.07	t-test	-0.13	22	0.89	0
A19	welch	10.51	12.86	<0.0001	-0.47	wilcox	58	-	0.44	-0.06
A20	t-test	4.59	22	0.0001	-0.13	t-test	-2.33	22	0.0291	0.01

Table S3. Minimum inhibitory concentration experiments

Exper	Time	Fluconazole level (µg)							
		0	0.5	1	4	8	32	128	512
1	Anc&Evol	x		x		x	x		
2	Anc	x		x					
3	Anc&Evol	x	x	x	x				
4	Anc	x		x	x	x	x		
5	Anc&Evol	x				x	x	x	
6	Anc&Evol	x					x	x	x
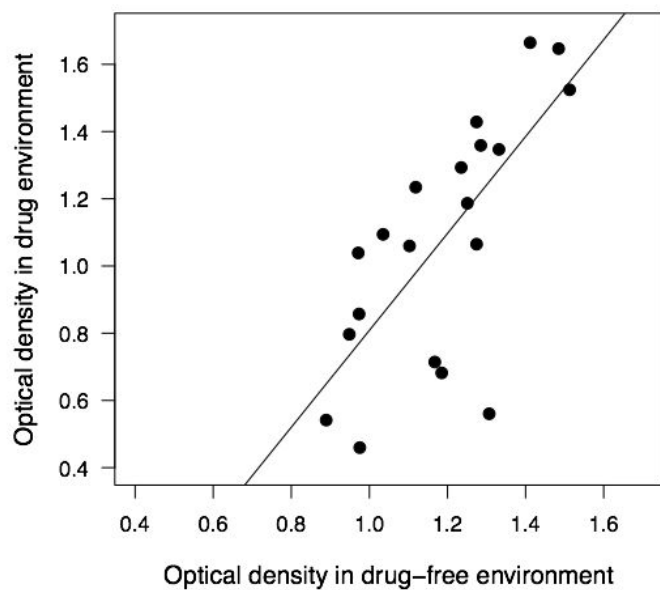
Figure S1. Evolved growth in a drug-free environment correlates with evolved growth in the drug environment at 24 h. Each point represents the mean of 12 replicates evolved from each strain background.
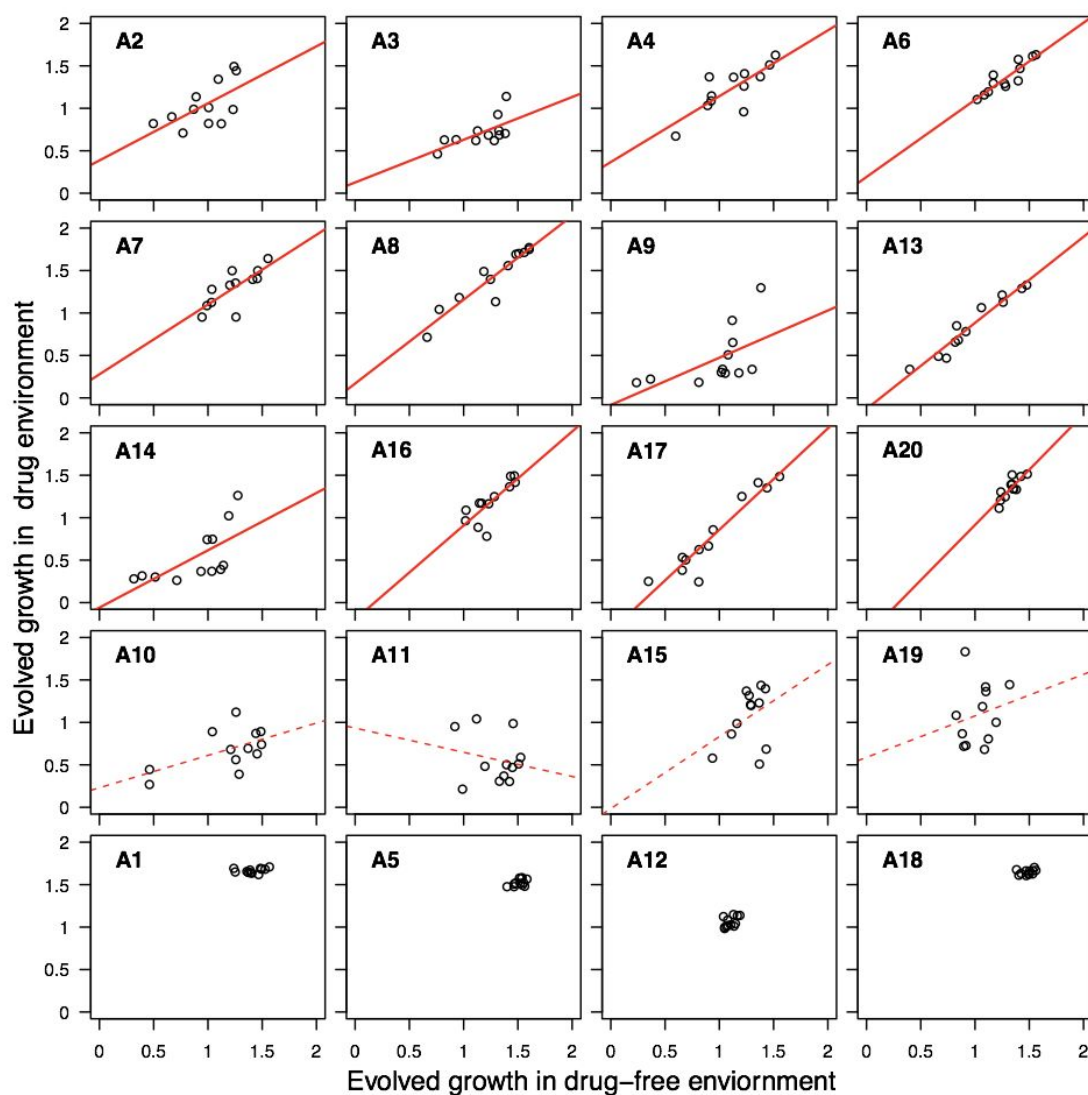
Figure S2. Evolved growth in a drug-free environment (YPD) is correlated with evolved growth in the evolutionary drug environment in many strain backgrounds. Growth in both environments was measured as optical density at 24 h. Each point represents an independently evolved replicate from the same strain background (indicated by the label in the top left corner of each figure panel). The solid red lines indicate a significant Spearman correlation test, dotted lines are for insignificant correlations. The replicate lines from the four strains plotted on the bottom (A1, A5, A12 and A18) were not variable enough to test for a correlation.
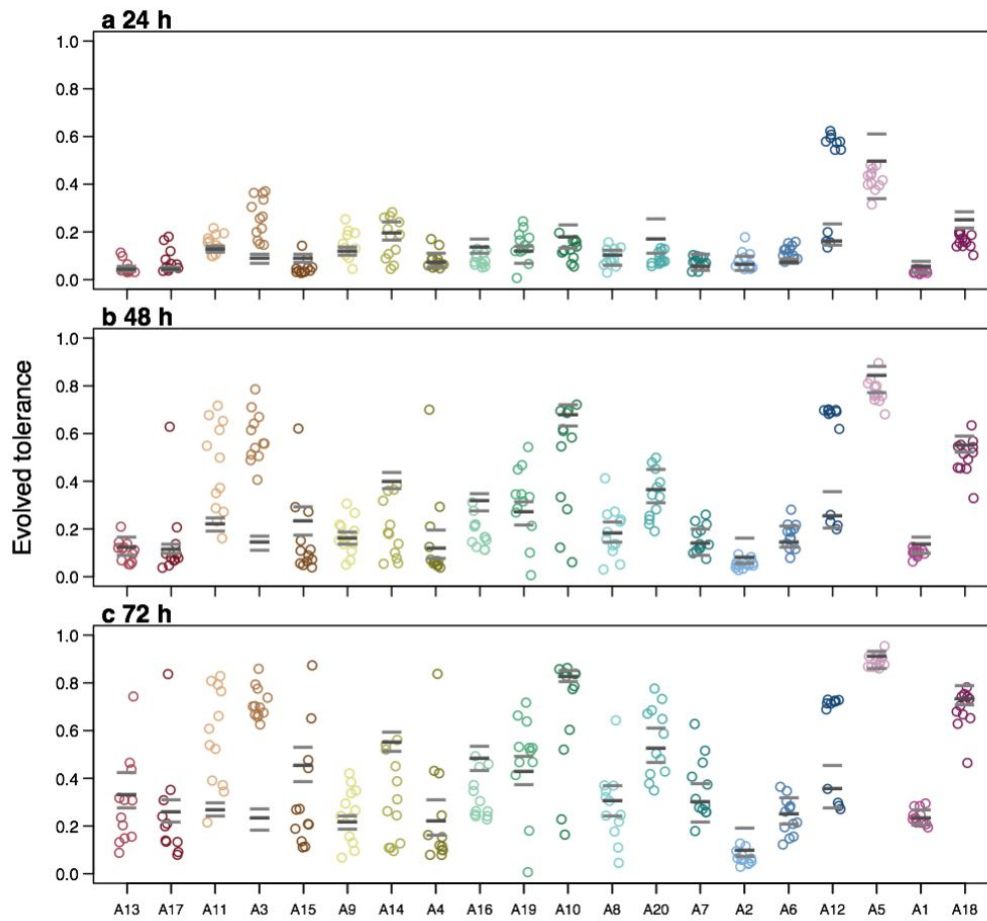
Figure S3. Tolerance of evolved strains measured at a) 24 h, b) 48 h, and c) 72 h.
Tolerance was measured as the growth observed in supra-MIC levels of fluconazole (as appropriate to each replicate) normalized to the growth in the absence of drug. Strains are arranged on the x-axis (and coloured) by initial growth in the evolutionary environment. Each point represents an individually-evolved replicate line. The black line indicates the median ancestral level and the two grey lines indicate the upper and lower bounds of twelve measured ancestral replicates.
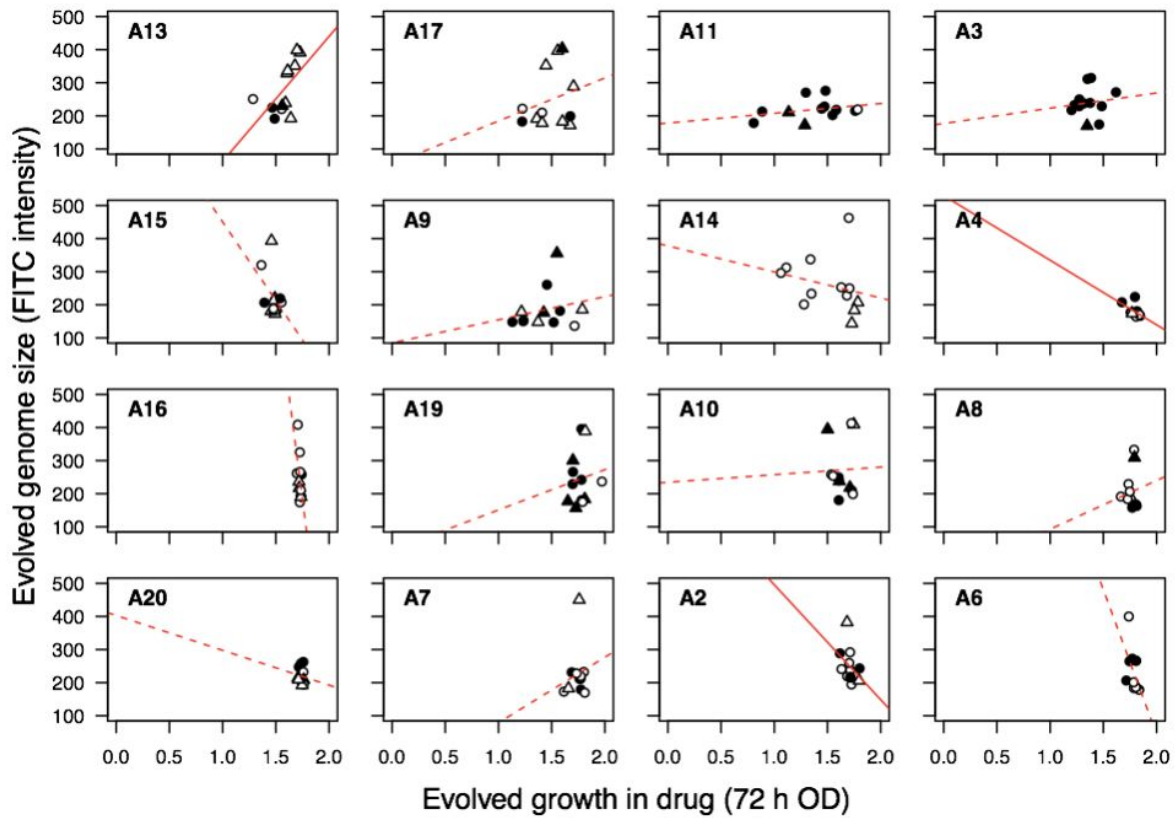
Figure S4. Evolved growth at 72 h and evolved genome size are not consistently correlated. Growth was measured as optical density after 72 hours in low drug. Evolved genome size indicates the most prominent G1 peak; when multiple G1 peaks were present this is indicated with a triangle symbol. Solid lines indicate a significant Spearman correlation test. Each point represents an independently evolved replicate line.
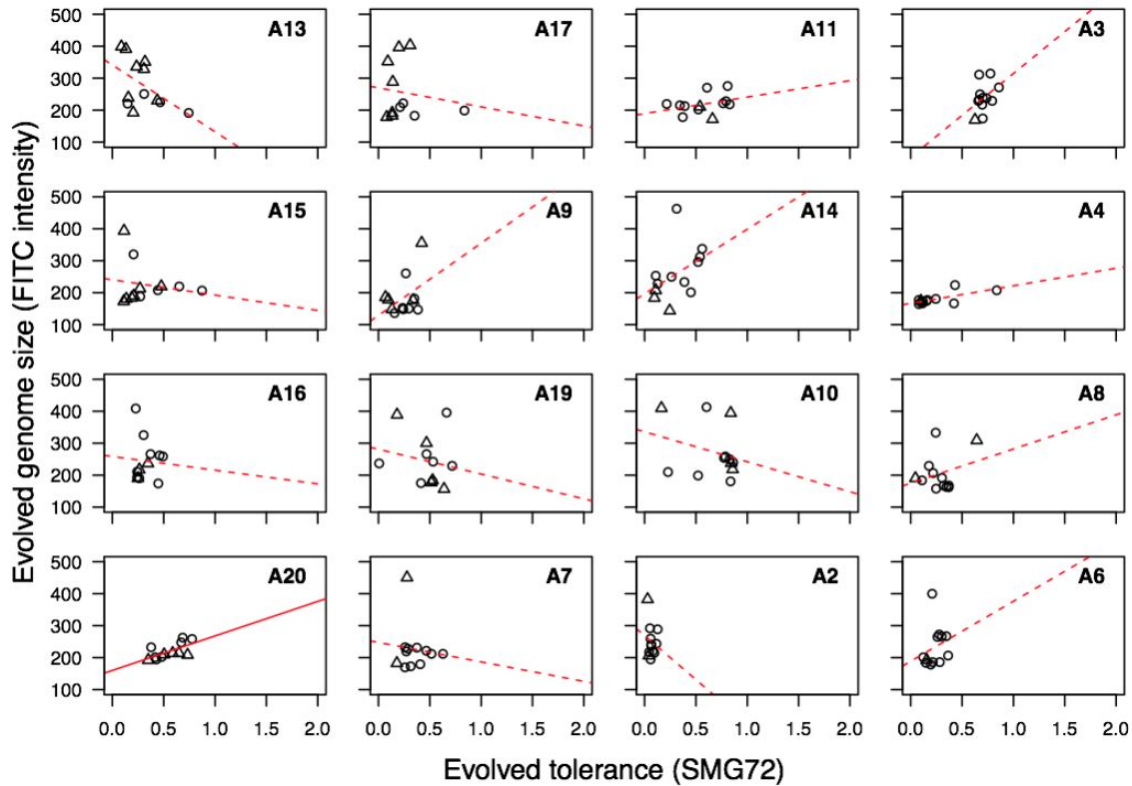
Figure S6. Evolved tolerance at 72 h and evolved genome size are only correlated in a single strain background. Each point represents an independently evolved replicate line. Tolerance was measured as SMG72. Evolved genome size represents the most prominent G1 peak; when multiple G1 peaks were present this is indicated with a triangle symbol. A solid line indicates a significant Spearman correlation test.