Ancestral fitness and genetic background influence diversity acquired during adaptation to low drug concentration in *Candida albicans*

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Abstract

Genetic background is likely to play a large role in the link between genotype and phenotype for *de novo* acquired mutations. In the context of drug resistance acquisition, an inherently evolutionary process, genetic background may directly determine the nature or scope of available mutations and/or the evolutionary potential of a strain acquiring drug resistance. We evolved replicates of twenty different *Candida albicans* strains for 100 generations to a low concentration of the common antifungal drug, fluconazole. We found that the majority of replicates rapidly increased in fitness in the evolutionary environment, and the degree of improvement was inversely correlated with ancestral strain fitness under those conditions. Unexpectedly, very few replicates in any genetic background acquired *bona fide* drug resistance. Rather, changes in drug tolerance (slow subpopulation growth at high concentrations of drug) and karyotype (aneuploidy and ploidy) were extremely prevalent, yet strain-specific. Importantly, low ancestral strain growth in low drug was correlated with high levels of evolved variation among replicates in growth, tolerance, and karyotype. This provides a link between strain background, mediated in part through ancestral growth ability, and the degree of acquired genotypic and phenotypic diversity among replicates during adaptation to low drug. Our results also highlight that caution must be exerted in genetic and evolutionary studies conducted with a single strain, as findings in a single background may not be representative or capture the diversity of possible responses when the diversity of a species is considered.

Introduction

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). By 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). Similarly, when human disorders are caused by variation in a single gene (monogenic traits), background-specific modifiers can lead to significant clinical phenotypic heterogeneity (i.e., variable expressivity, Cooper et al. 2013, and references within).

The acquisition of drug resistance is an inherently evolutionary process, and genetic background may directly determine the nature or scope of available mutations and their evolutionary potential. Typically, alteration of only a finite number of cellular mechanisms can lead to drug resistance, defined as the ability of a strain to grow at drug concentrations above a specific, clinically-established, minimal inhibitory concentration (MIC) of the drug. However, even for well-studied pathogen/drug pairs, the factors that influence the rate of resistance have not been well elucidated. Thus, understanding the processes that lead to the appearance and spread of reduced drug susceptibility can help inform clinical decisions.

The acquisition of drug resistance differs fundamentally between fungal and bacterial pathogens (Levin and Bergstrom 2000). Eukaryotic pathogens predominantly evolve through genomic mutations that are acquired *de novo* or transmitted vertically. Furthermore, fungal genomes are labile, as karyotypic variation, whether via changes in ploidy (the number of homologous chromosome sets) or aneuploidy (chromosome gain or loss) is frequently observed in laboratory strains subjected to stress (Wellington and Rustchenko 2005; Bouchonville et al. 2009) or passaged through mice (Forche et al. 2009, 2018). Karyotypic variation has also been detected in clinical isolates of both *C. albicans* and *S. cerevisiae* (Ford et al. 2015; Zhu et al. 2016). The rate of change in karyotype occurs much more frequently than the rate of point mutations (Zhu et al. 2014), is elevated in the

presence of drug stress, and can be rapidly lost in the absence of drug stress (Perepnikhatka et al. 1999; Gerstein et al. 2015; Hickman et al. 2015; Anderson et al. 2017; Hirakawa et al. 2017). Hence, karyotpyic variation provides eukaryotic pathogens with a rapid and potentially reversible response to drug stress.

In pathogenic fungi, a mechanistic link between karyotypic variation and drug resistance has been determined in some cases. In *Candida albicans*, the most prevalent human fungal pathogen, isolates that carry isochromosome 5L (i[5L]) are resistant to the antifungal drug fluconazole, because i[5L] carries two extra copies of two genes, *ERG11*, which encodes the target of fluconazole,and *TAC1*, which encodes a transcription factor that positively regulates efflux pump levels (Selmecki et al. 2006, 2008). Similarly, *Cryptococcus neoformans* isolates aneuploid for chromosome 1, which carries both the *ERG11* ortholog and *AFR1,* encoding a drug efflux pump, are also transiently fluconazole-resistant (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 (e.g., chromosome 4 in *C. neoformans,* Sionov et al. 2010; Ngamskulrungroj et al. 2012*)*, chromosomes 3, 4, and 6 in *C. albicans* (Perepnikhatka et al. 1999; Anderson et al. 2017; Hirakawa et al. 2017). Intriguingly, certain aneuploidies appear to be beneficial in some backgrounds but not in others (e.g., Anderson et al. 2017; Hirakawa et al. 2017). Intriguingly, exposure to fluconazole and other azole drugs also causes karyotypic change by inducing deviations in cell cycle regulation (Harrison et al. 2014; Altamirano et al. 2017). Hence fluconazole can both cause and select for karyotypic variation and when present in fluconazole-resistant isolates, it can be difficult to determine whether karyotypic variation is causative or merely correlative.

The phenomenon of drug tolerance and may play an important role in fungal response to drug (Rosenberg et al. 2018). Tolerance has been broadly defined as the ability of cells to grow at drug concentrations above the minimum inhibitory concentration (MIC) (Fridman et al. 2014; Delarze and Sanglard 2015). Importantly, strains that exhibit tolerance are 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). We recently developed techniques to quantify the size of the subpopulation that is able to grow slowly at supra-MIC drug concentrations and find that drug tolerance does not correlated with drug resistance (Gerstein et al. 2016; Rosenberg et al. 2018).

Here, we set out to explore the interplay between genetic background, *bona fide* resistance, tolerance, and karyotypic variation in a broad range of *C. albicans* strains. We evolved replicates from twenty different *C. albicans* strains for 100 generations to a low concentration of fluconazole. Evolving many replicates simultaneously facilitates the distinction between common and rare evolutionary trajectories. Only a handful of experimental evolution experiments have been conducted with *C. albicans* (Cowen et al. 2000, 2001; Selmecki et al. 2009; Anderson et al. 2015), and none have examined whether drug tolerance changes upon sustained exposure to drug.

The majority of replicates rapidly increased growth in the low drug, evolutionary environment, with the degree of improvement inversely correlated with ancestral strain growth in the low drug. Unexpectedly, very few replicates from any background acquired *bona fide* drug resistance. By contrast, 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. Importantly, ancestral strain growth was directly correlated with evolved variation in growth, tolerance, and karyotype, providing a link between strain background (mediated at least partially via ancestral growth) and the degree of parallelism among replicates during adaptation to low drug.

Results

We examined the evolution of 240 replicates from twenty different clinical strains of *C. albicans* passaged in fluconazole (1 μg/mL FLC) for ~100 generations (20 strains x 12 replicates per strain). The strains were chosen to span the phylogenetic diversity of the species (Ropars et al. 2018), and vary in mating type zygosity, geographic origin and site of isolation (Table 1). Growth of ancestral and evolved replicates was assessed at two time points: optical density (OD at A_{600}) at 24 h is consistent with clinical assessment of drug resistance, and should correlate with growth rate in the first 24 h (Gerstein et al. 2012); OD at 72 h correlates with population size at the time of transfer. We primarily use growth as a proxy for fitness in the evolutionary (low drug) environment, as this encompasses the ability to convert nutrients from the environment into cellular biomass. Drug resistance (MIC_{50}) was measured using standard liquid broth microdilution methods at 24 h while tolerance, measured as growth above the MIC_{50} in a liquid assay (SMG: supra-MIC_{50} growth, Rosenberg et al. 2018) was measured at both 24 and 72 h.

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 evolved replicates acquired significantly higher growth ability than their respective ancestral strains, when grown for 24 or 72 h in the evolutionary environment (1 µg/mL fluconazole; Figure 1; detailed statistical results in Table S1). The major exception was replicates from ancestrally-resistant strain A12, which had the highest ancestral MIC_{50} of 32 µg/mL, well above the drug concentration used here. A12 evolved significantly lower 24 h growth, and did not change in average growth at 72 h. Four strains with intermediate levels of growth in the low drug evolutionary environment (A2, A9, A10 and A14) also did not acquire improved average growth among replicates at 24 h, although on average all had significantly increased growth at 72 h.

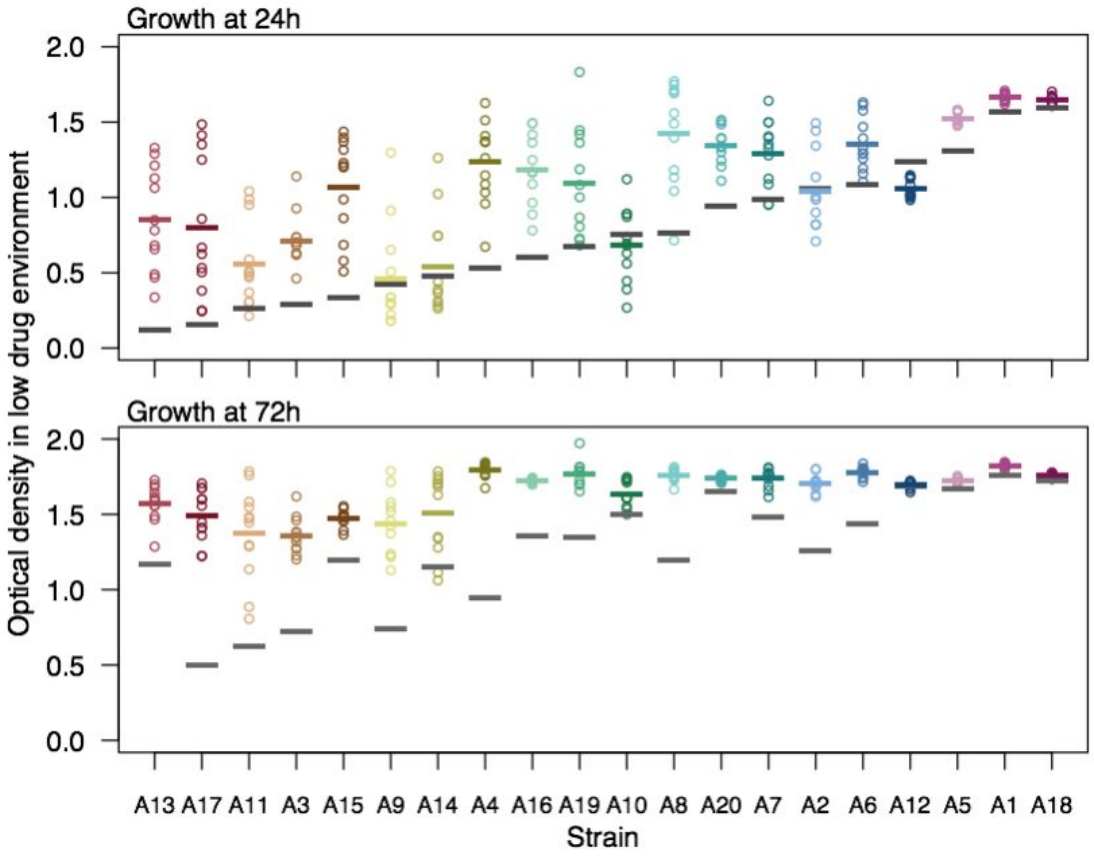

Figure 1. The majority of evolved replicates improved growth in the evolutionary environment after ~100 generations of evolution. Fitness was measured as optical density in YPD + 1 µg/mL FLC, the evolutionary environment after 24 h (top) and 72 h (bottom). Strains are ordered by ancestral growth in low drug at 24 h. The ancestral growth is indicated for each strain by a grey bar, representing the median growth among 12 replicate ancestral measurements. Each open point represents one of twelve replicates evolved for each ancestral genotype, the coloured bars indicate median evolved growth for visual comparison to ancestral growth.

The mean degree of growth improvement among replicates was strongly influenced by ancestral strain growth, 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: $F_{1, 13} = 0.25$, $p = 0.63$). There was a very strong correlation between the change in growth (improvement in growth between evolved and ancestral replicates) and the ancestral growth 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 can be seen, however, by strains that deviated considerably from the correlation line of best fit (Figure 2).

The variance among evolved replicates from each strain reflects stochasticity in the evolutionary process. There was a significant negative correlation between the variance in evolved growth and the degree of growth improvement: 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, after 100 generations of evolution in low drug, 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.

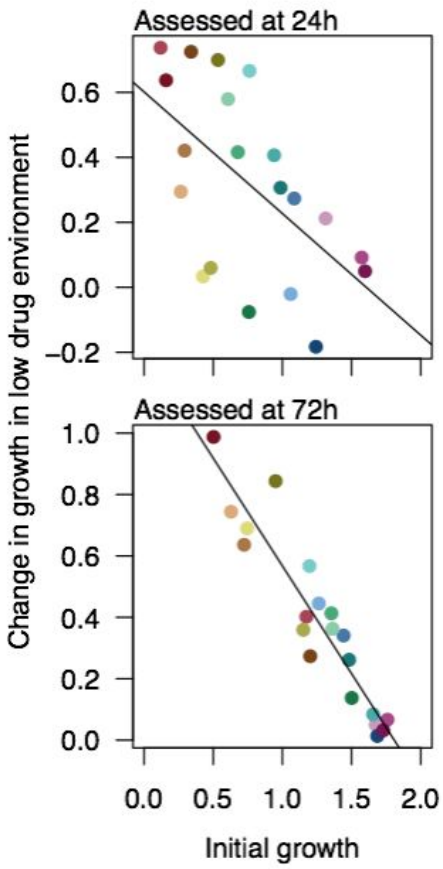

Figure 2. Significant negative correlation between ancestral growth and change in growth. Growth was measured as optical density in YPD+1 µg/mL FLC, the low drug evolutionary environment after 24 h (top) and 72 h (bottom). The colour matches Figure 1, based on ancestral growth (low ancestral growth strains 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

Mutations that fixed in bacteria in response to antimicrobial drugs tend to result in a tradeoff with growth in the absence of drug (i.e., a fitness cost), although several drug classes and bacterial species do not incur a cost on average (Melnyk et al. 2015), and references within). Mutations that provide an advantage to fungi exposed to antifungal drugs appear to follow a similar pattern of species/drug specificity on tradeoffs. For example, mutations that confer an advantage to high concentrations of nystatin in *Saccharomyces cerevisiae* incur a significant cost (Gerstein et al. 2012), yet mutations that confer an advantage to high concentrations of fluconazole in *Candida albicans* incur no appreciable cost (Anderson et al. 2003; Selmecki et al. 2009). Furthermore, it remains an open question whether mutations that provide an advantage to low concentrations of drug should be expected to show a similar degree of cost as mutations that enable growth in higher concentrations of drug.

After 100 generations of evolution to a low concentration of fluconazole, the majority (80%) of evolved replicates grew more slowly than the ancestral replicates at 24 h in a drug-free environment (standard lab YPD, the replete base medium for the evolutionary environment without the addition of fluconazole; Figure 3). The average growth reduction between evolved and ancestral replicates among significant strain background was 21%. The exceptions were strain A5, where the majority of evolved replicates showed a significant growth improvement (6% improvement), and strains A8, A10 and A11, where evolved replicates had no significant change on average (Table S2). When growth was measured at 72 h, although there was a statistically significant change in evolved compared to ancestral growth in some backgrounds, the actual change was much lower than at 24 h or in the evolutionary (drug) environment (Table S2; strains with a significant improvement at 72 h growth, average growth improvement of 1%: A1, A4, A5, A10, A11, A12, A20; evolved strains with significantly reduced growth, average growth reduction of 5%: A3, A13, A14, A15, A17). Hence, the majority of replicates evolved in 1 μg/ml fluconazole exhibit slower initial growth in a drug-free environment, yet the majority reached the same or slightly higher growth by 72 h. This points to an increased amount of time spent in lag phase in the drug-free environment.

Notably, we found a significant positive relationship between mean growth in the low drug and drug-free environments among strain backgrounds at 24h (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 growth in both drug-free and low drug environments; 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. 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.

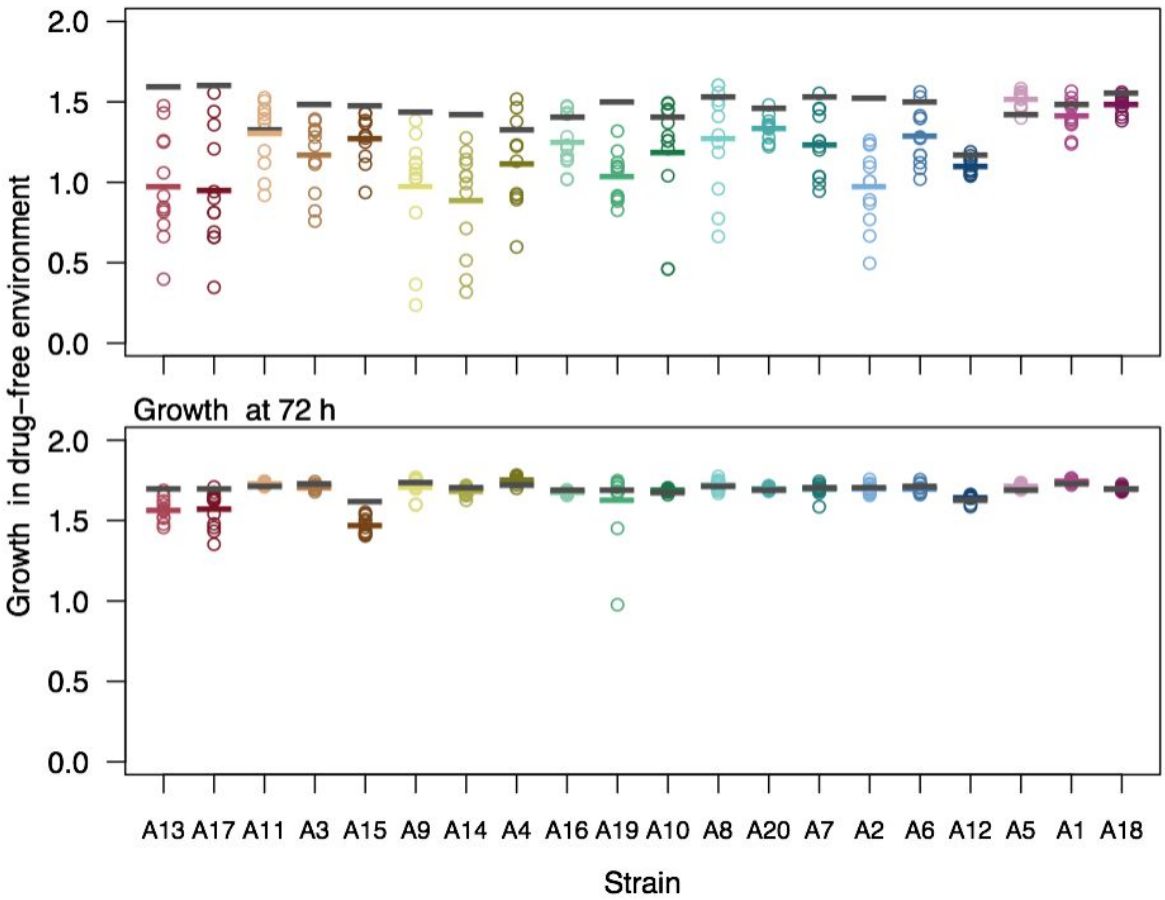

Figure 3. Growth in a drug-free environment. Growth was measured as optical density in YPD, the standard lab rich medium, after 24 h (top) and 72 h (bottom).Strains are ordered by ancestral growth ability in the low drug evolutionary environment. Ancestral growth in YPD is indicated by a grey bar. Each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

Increases in *bona fide* drug resistance were rare and changes in drug tolerance were common

Drug susceptibility/resistance is a concentration-dependent property of individual strains that is measured as the MIC_{50} using broth microdilution assays. We next asked if prolonged exposure to 1 μg/mL fluconazole selected for decreased susceptibility (increased resistance). Only 18 of the 240 evolved replicates had an increase in MIC_{50} beyond the evolutionary level, and these replicates hailed from only seven of the 20 strain backgrounds (Figure 4). In the majority of these replicates, MIC_{50} increased only slightly, to 4 μg/mL (five replicates from strain A3, four replicates from A8, and one replicate from A4 and A7). The acquisition of clinical resistance ($MIC_{50} \geq 8$ μg/mL at 24 h, (Fothergill et al. 2014) arose in replicates from only three backgrounds: six from strain A5 (which had an initial MIC_{50} of 4 μg/mL) and one each from strains A17 and A19, two backgrounds with a very low initial MIC_{50}. Interestingly, MIC_{50} decreased to 1μg/mL in some of the replicates from the five strain backgrounds with ancestral MIC_{50} values above 1 μg/mL (two replicates from A5, eight replicates from A12, all 12 replicates from A20, Figure 4). This suggests a fitness cost of ancestrally-high MIC_{50} during prolonged growth in low concentrations of drug in some, but not all, strain backgrounds.

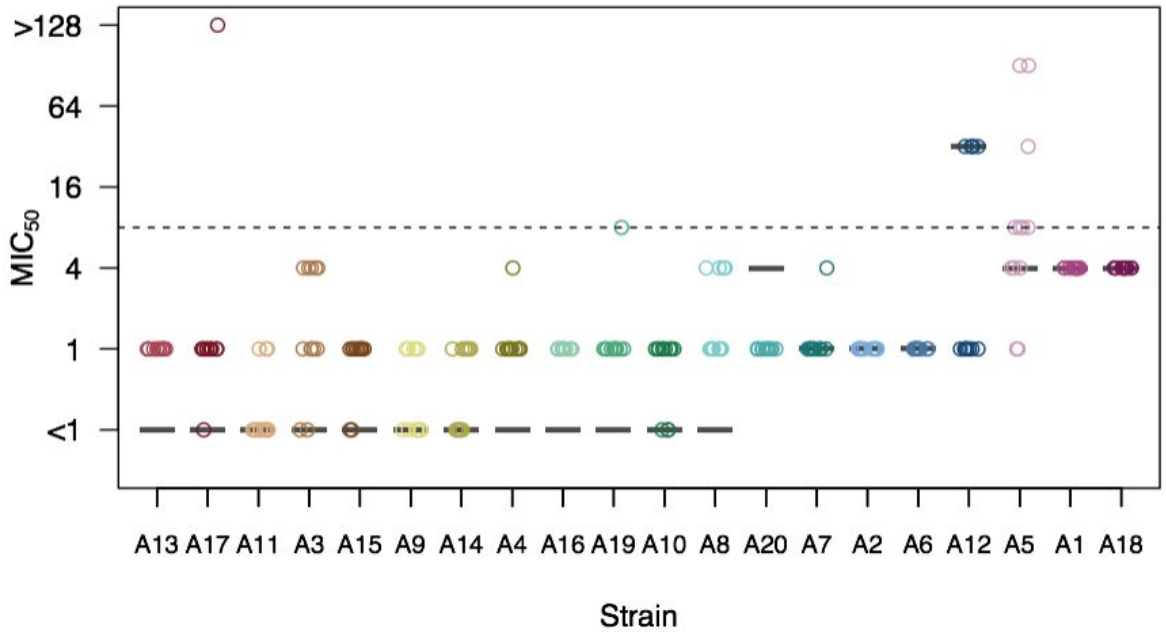

Figure 4. The majority of evolved replicates did not acquire resistance. Drug resistance was measured through broth microdilution assays. The clinical cutoff for resistance at 8 µg/mL fluconazole is indicated by the dashed line. Strains are arranged on the x-axis (and coloured) by initial growth in the evolutionary environment. Each point represents an individually-evolved replicate line.

In contrast to the rare evolution of increased MIC_{50}, increases and decreases in tolerance (SMG) were prevalent among evolved replicates (Figure 5, Figure S3). Approximately one third of the evolved replicates increased in tolerance measured at 24 h (72 replicates from 13 different strain backgrounds) and 72 h (73 replicates from 13 strain backgrounds), while a similar number decreased in tolerance at both time points (24 h: 79 replicates from 17 strain backgrounds, 72 h: 91 replicates from 17 strain backgrounds). Consistent with a previous examination of SMG (Rosenberg et al. 2018), the magnitude of tolerance change increased sharply between 24 and 48 h of drug exposure. Thus, to be consistent with the timing of our transfers and growth measurements, we primarily considered SMG at 72 h to represent the tolerance in evolved replicates (SMG 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). Intriguingly, 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 influenced by 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: $F_{1,14} = 2.53$, $p = 0.13$). However, a significant positive correlation was recovered between variation in evolved growth and the variance in tolerance (Spearman's rank correlation: growth measured at 24 h: S = 588, p-value = 0.012, rho = 0.56; this relationship was statistically not-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 initial growth, influenced the evolved variation among replicates in the evolution of tolerance as well as resistance.

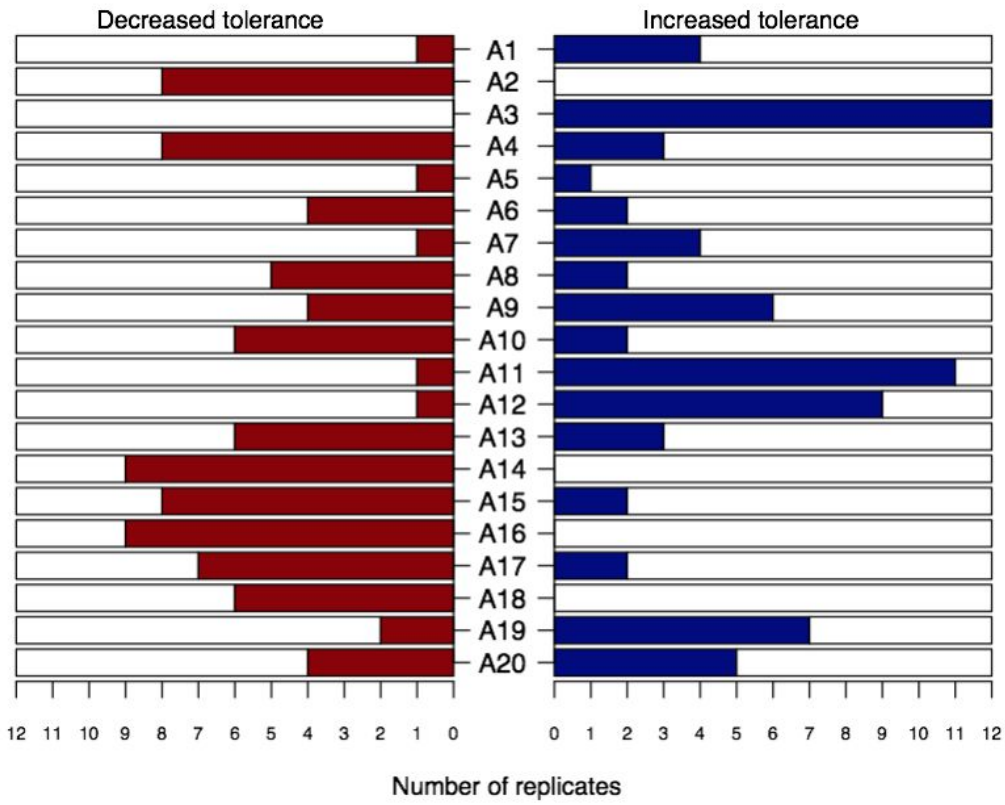

Figure 5. Many evolved replicates decreased (red) or increased (blue) in tolerance. Tolerance was measured as the growth observed in supra-MIC levels of fluconazole normalized to the growth in the absence of drug after 72 h ("SMG72"). Strains are arranged in order of increasing growth in the evolutionary environment at 24 h (top-bottom here corresponds to left-right in Figure 1).

Genome size changes are pervasive

Given that exposure to fluconazole induces the formation of tetraploid, and subsequently aneuploid, cells (Harrison et al. 2014), and that specific aneuploidies can be selectively advantageous (Selmecki et al. 2006; Yang et al. 2013; Anderson et al. 2017), we asked if evolved replicates exhibited altered ploidy and, if so, whether the degree to which ploidy was changed was a strain background-dependent characteristic.

Table 2. Welch two sample t-test comparing 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	$t_{11.5} = 5.91$, $p < 0.0001$

Across the majority of strain backgrounds, evolved replicates underwent a significant increase in median genome size (Figure 6A, Table 2). The three exceptions were A12, the resistant strain with the highest ancestral MIC_{50} (MIC_{50} = 32 μg/mL) and high initial growth in the evolutionary environment; and A4 and A9, two strains with MIC_{50} values below < 1 μg/mL and intermediate initial growth in the evolutionary environment. Strain A9 was also one of the intermediate growth strains that did not acquired higher growth at 24 h in low drug.

The coefficient of variance for ploidy level also increased among replicates 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 genome compositions.

Importantly, the four strains that did not exhibit obvious variation in the evolved replicates were the four parental strain backgrounds that had the highest initial growth levels in low drug and MIC_{50} above 1 μg/mL: strains A1, A5, A12 and A18. This pattern was readily apparent from the flow cytometry traces (Figure 6C). When these four strains were removed from the analysis, median evolved genome size was not correlated with ancestral low drug growth (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 karyotypic changes among strain backgrounds.

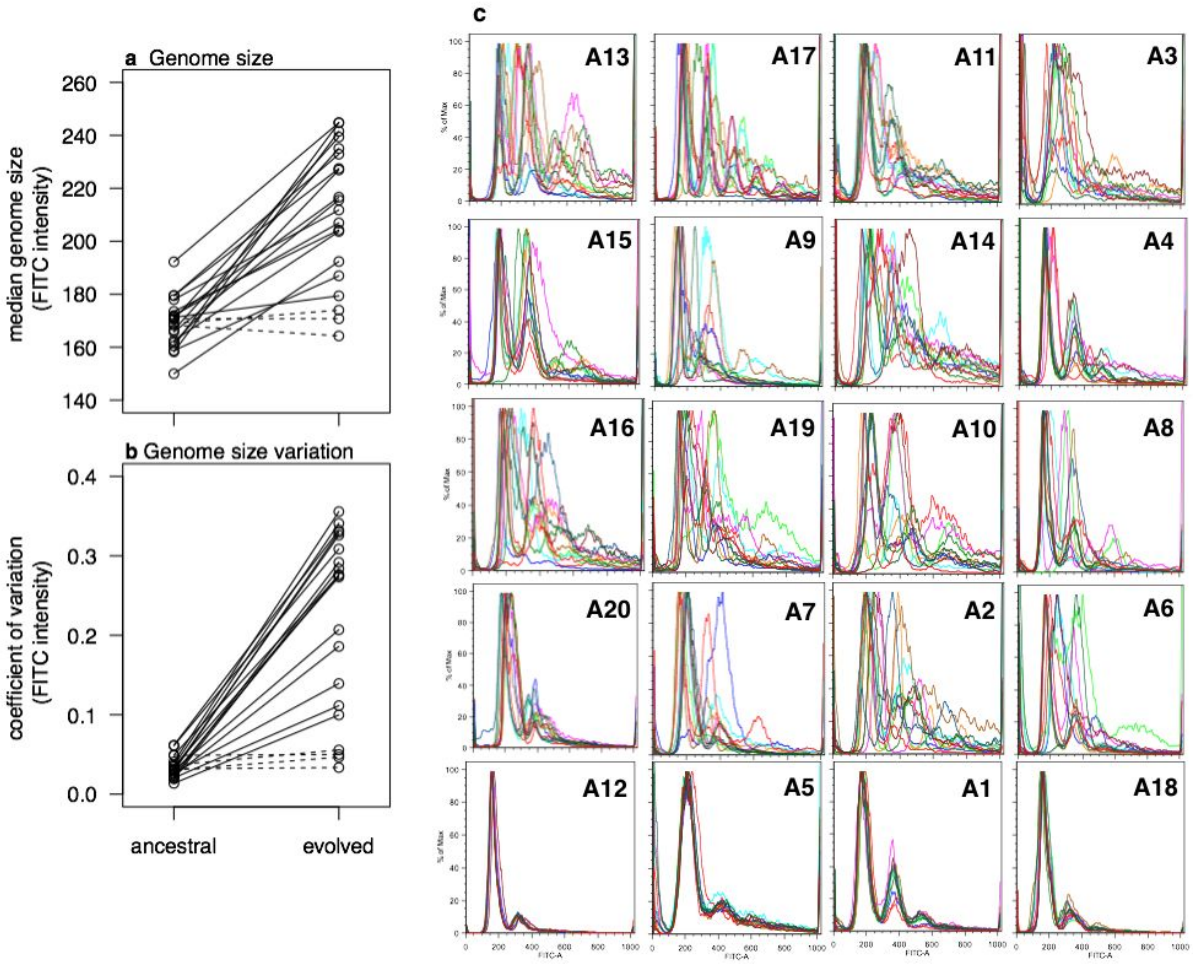

Figure 6. Genome size and variation in genome size increased after evolution to low drug. a) Median genome size, and b) coefficient of variation (CV; i.e., variability among replicates) in each strain background. A dashed line indicates a non-significant change between ancestral and evolved replicates. c) Flow cytometry traces of each replicate evolved line, arranged by ancestral strain growth in low drug 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. We examined the relationship between evolved genome size and evolved growth for replicates from each of the 16 strain backgrounds that evolved variable genome size. Evolved growth at 24 hours and evolved genome size were significantly correlated in five strains: in A6, A8, A10 and A16 there was a negative correlation (replicates that increased in genome size tended to have less growth at 24 h), while in strain A13

there was a positive correlation (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 tolerance (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, although there was no direct link between evolved genome size and evolved growth in most strain backgrounds, we again found that the variability among replicates is consistently larger in some strain backgrounds than others, mediated in part by ancestral strain growth in the evolutionary environment.

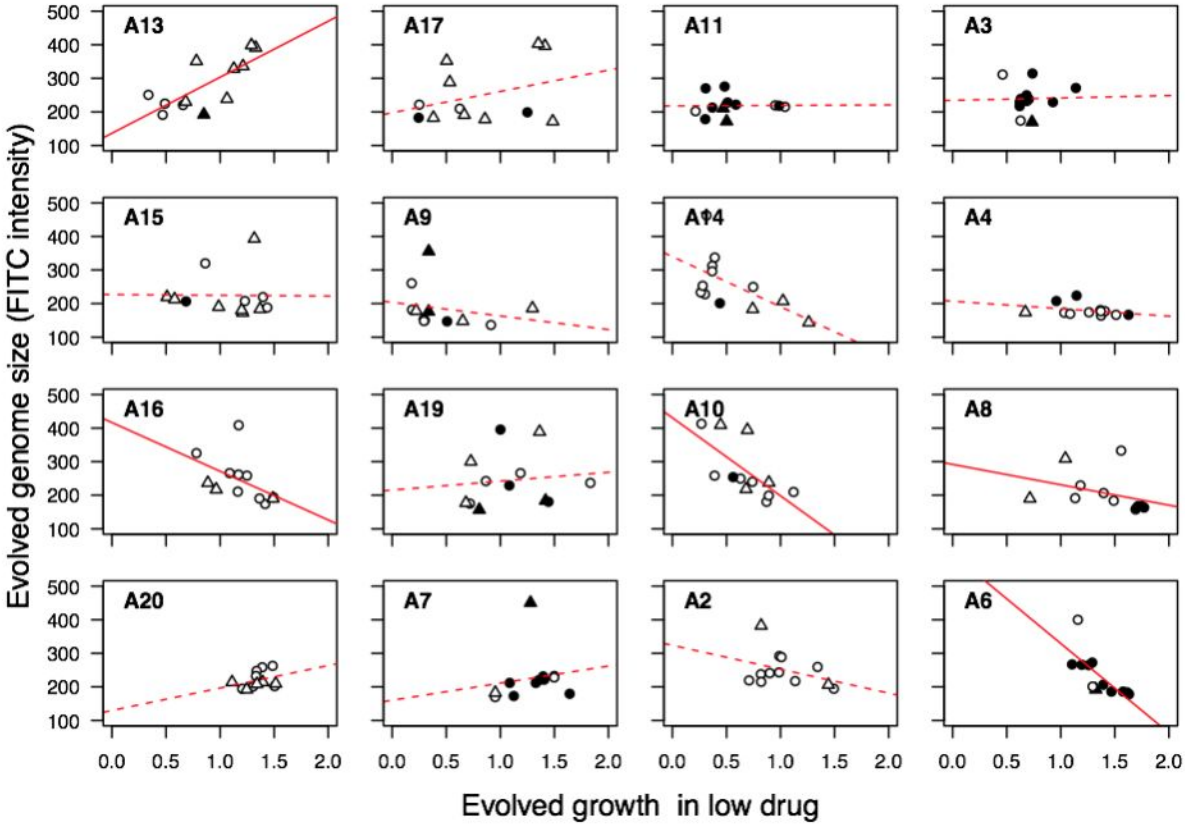

Figure 7. Evolved growth and evolved genome size are not consistently correlated. Growth was measured as optical density after 24 hours in low drug. Evolved genome size indicates the most prominent G1 peak; when multiple G1 peaks were present this is indicated with a triangle symbol. Solid lines indicate a significant 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 that is dictated in part by the specifics of the population and the evolutionary environment. Few experiments, however, have directly quantified the influence of genetic background on the nature and rate of adaptation. Here, we evolved 12 replicates from 20 *C. albicans* strains to a relatively low level of fluconazole (1 µg/ml fluconazole) that is nevertheless above the ancestral MIC_{50} of the majority of strains. The short period of laboratory evolution (100 generations) was chosen to capture the first one or several beneficial mutations in each replicate, based on the rationale that mutations that increase MIC_{50} in a clinical setting would typically be the first beneficial mutations that confer the ability to grow in the presence of the drug stress. We found that improvements in growth in the evolutionary environment and changes in drug tolerance were far more prevalent than changes in MIC_{50}.

The majority of replicates rapidly increased in growth, and the mean growth improvement was negatively correlated with ancestral strain growth. Similar results from experimental evolution studies were reported for eight divergent bacteriophage genotypes (Rokyta et al. 2009) and for five different *E. coli* strain backgrounds (-G. Moore and Woods 2006). The pattern of diminishing-returns for fitness is also consistent with a suite of related microbial experiments: a negative correlation between ancestral fitness and rate of adaptation in experimental evolution studies that were initiated with closely related strains of the same ancestral background that differed 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).

Of note, in our study the correlation between ancestral growth and change in growth was strongest when growth was measured at 72 hours (adjusted r-squared from the log-log regression = 0.96) compared to 24 hours (adjusted r-squared = 0.72), the time when MIC is clinically determined. 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 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: A2, A3, A9, A10, A12, A14. Nothing that we know about this strain set stands out—they are from different clades, include both homozygous and heterozygous mating loci, and were isolated from diverse geographic and clinical locations. Nevertheless, 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 time at which growth is measured affects interpretations of the role of ancestral fitness.

In contrast to the rapid growth improvements observed in the evolutionary environment, very few (<5%) of replicates with initial MIC_{50} at or below 1 μg/mL fluconazole increased their MIC_{50} to > 1 μg/mL. Thus, there was only a small window of MIC_{50} improvement that did not extend beyond the concentration of drug that the replicates were evolved in. The influence of subinhibitory drug concentrations on MIC_{50} remains under-studied in fungi. In *Sclerotinia sclerotiorum,* a plant pathogenic fungus, there was not a consistent relationship between of increased resistance after repeated exposure to sublethal concentrations of different antifungal drugs. In three drugs (azoxystrobin, boscalid, thiophanate methyl) approximately half of strains increased in MIC_{50}, while in two additional drugs (pyraclostrobin, iprodione) almost no strains acquired increased MIC_{50} (Amaradasa and Everhart 2016). Many studies in bacteria have, however, 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; McVicker et al. 2014; Mira et al. 2015; Lundström et al. 2016). This highlights the important influence of different drugs as well as the potential differences between different species on the breadth and molecular influence of drug resistance mutations.

Strikingly, a third (22 of 60) of evolved replicates with ancestral MIC_{50} above the evolutionary environment evolved to have 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 to 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 trade-off exists between alleles that provide high fitness in low drug and those that provide high fitness in high drug, in at least some strain backgrounds. Furthermore, it demonstrates that *C. albicans* MIC_{50} can rapidly decrease upon exposure to low concentrations of fluconazole, a result that future experiments in a clinical context (e.g., *in vivo* evolution in mice) will seek to replicate.

We also found that drug tolerance, the ability of a subpopulation to grow slowly at high drug concentration, often evolves during exposure to drug. Intriguingly, over half of the evolved replicates had altered tolerance levels with both increased and decreased tolerance detected. This indicates that the ability of a subpopulation to grow in concentrations of drug 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. For example, 12/12 replicates from strain A3 increased in tolerance, while 9/12 replicates from strains A14 and A16 decreased in tolerance (with no change in the other three replicates). Other strains exhibited variability among replicates (e.g., strain A9—four replicates decreased while six replicates increased tolerance). The lack of correlation of evolved tolerance with ancestral growth levels, MIC_{50}, or known strain characteristics (i.e., *MAT* genotype or clade), suggest that the evolution of tolerance is not easily predicted based on resistance or fitness and may well depend on the genetic background of a given strain.

The appearance of karyotypic variability among evolved replicates also was strain-dependent. The four strains with the highest initial growth in low drug (all with ancestral MIC_{50} > 1 μg/mL) had stable genomes with no change in their variability after evolution. By contrast, karyotypic variability increased significantly for all other strain backgrounds. There was also variation in the

variability among replicates: replicates from some backgrounds were more variable than others, and the degree of variability in karyotype among replicates correlated significantly with variability both in evolved growth and in evolved tolerance. There was not, however, a consistent correlation between evolved ploidy level and evolved growth, or evolved ploidy level and tolerance. Only in one strain background did increase in genome size and increased growth correlate significantly (i.e., increased growth was associated with the elevation in ploidy level), and in three backgrounds ploidy level and increased growth were inversely correlated. Thus, some strain backgrounds were more likely to yield evolved populations with variable phenotypes than others, regardless of the phenotype being measured, yet the actual phenotype parameter values were not necessarily correlated with each other or even in the same direction.

High concentrations of fluconazole (10 μg/ml) were previously found both to cause and select for aneuploidy (Selmecki et al. 2006; Harrison et al. 2014). Here, a 10-fold lower concentration of fluconazole also rapidly caused aneuploidy to arise within populations, presumably via a similar mechanism. The variation that remains to be sampled seem to be largely neutral. Of note, the concentration of drug used (low or high) must be considered relative to the initial strain growth/MIC_{50}: the degree of stress that a strain encounters is relative, such that a "low" concentration of drug in one strain (e.g., with an MIC_{50} well above that concentration) likely is sensed physiologically as a high concentration of drug for a different strain (with an MIC_{50} below that concentration). An indicator of this difference is the low evolved ploidy variability observed for strains with initial MIC above the drug evolutionary environment. Nonetheless, 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 low drug growth and tolerance are distinct from those that provide resistance to higher concentrations of drug. Furthermore, it suggests that many more genes and genetic pathways likely influence growth in a low fluconazole concentration and fluconazole tolerance than the small number of genes that directly affect *bona fide* fluconazole resistance.

Conclusion

Experimental evolution studies provide an extremely useful framework to isolate important factors that influence adaptation. Here we evolved replicates from 20 different strain backgrounds to probe the importance of strain background on

Candida albicans evolution to a low drug environment (1 μg/mL fluconazole). We found that, in accordance with theoretical predictions, genetic background had a significant influence on the rate of adaptation, mediated in part through ancestral fitness relative to the selective conditions used. Fitness improvements were largely restricted to the low drug environment used in the evolution experiment, indicating that adaptation to low concentrations of fluconazole often does not select for resistant mutants. Indeed, a number of replicates from strains with initially high MIC evolved an increase in drug susceptibility. Genome size changes were prevalent among replicates evolved from strains that had ancestral MIC below the drug concentration used in the evolutionary environment, yet was largely absent from ancestral strains with higher MIC. 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. Strain background is thus an important factor that needs to be taken into account explicitly when making generalizations about stress response.

Materials and Methods

Strains

Twenty diploid clinical strains of *Candida albicans* were selected to represent the phylogenetic diversity of the species, with at least four strains from each of the four major clades that encapsulate ~70% of the typed *C. albicans* strains and nearly span the known phylogenetic diversity of the species (Odds et al. 2007; Ropars et al. 2018) and includes four commonly-studied laboratory strains (SC5314, FH1, DSY294, T188). For each clade, strains with both heterozygous (*MAT**a**/MAT*) and homozygous (*MAT**a**/MAT**a**, MAT* */MAT*) mating loci were chosen. The strains were chosen blind with respect to the initial minimum inhibitory concentration of drug (MIC). Full strain designations including clade designation, country of origin, and site of isolation were obtained from the original manuscripts (Table 1) and (Tavanti et al. 2005). Mating type genotype was confirmed by PCR with *MAT**a*** and *MAT* specific primers (*MAT**a*** F-TTGAAGCGTGAGAGGCAGGAG, *MAT**a*** R-GTTTGGGTTCCTTCTTTCTCATTC, *MAT* F-TTCGAGTACATTCTGGTCGCG, *MAT* 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) in the wild type *C. albicans* population (Delarze and Sanglard 2015). This concentration of drug was equivalent to the ancestral MIC of three strains, above the MIC for 11 strains and below the MIC for one highly resistant strain (MIC 32) and four additional strains (MIC 4) (Table 1).

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

From the overnight cultures, we froze 100 μL from each replicate in duplicate in 50% glycerol as the ancestral replicats. 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 20uL 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 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). This length of time was chosen predominantly to capture the first genomic mutations that arose and reached high frequency.

50μl of the evolved replicate cultures were frozen in duplicate in 50% glycerol after the third, fifth, eighth and tenth (final) transfer and maintained at -80 °C.

Resistance and tolerance

The initial susceptibility and tolerance of all strains was tested using broth microdilution liquid assays as minimal inhibitory concentration (MIC) and supraMIC growth (SMG), respectively. The liquid assay experiments followed the initial cell dilution regulations from the clinical CLSI M27-A guidelines, using 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. Due to capacity (with 20 strain backgrounds x 12 replicates) we were not able to assay all drug concentrations for all environments in each assay. A single measurement was taken for each replicate at each concentration of drug that was measured in a given experiment (see Table S4). For each replicate at each time point (ancestral and evolved), the data from all experiments was combined, with the median OD determined at each concentration of drug. 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. 2017)(Rosenberg et al. 2018)(Rosenberg et al. 2017). 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. Tolerance was assessed at 24, 48 and 72 h ("SMG24", "SMG48" and "SMG72" respectively). 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 used “growth” as a proxy measurement of fitness in the evolutionary (low 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 and is consistent with the clinical determination of drug resistance from standardized liquid assays.

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 *MAT* / 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.

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Supporting 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).

	Growth ability at 24 h					Growth ability at 72 h				
Line	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).

	Growth ability at 24 h					Growth ability at 72 h				
Line	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

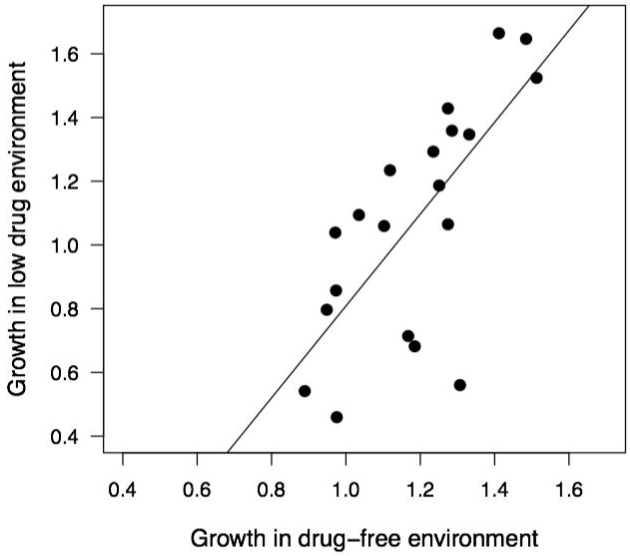

Figure S1. Evolved growth in a drug-free environment correlates with evolved growth in the low drug environment at 24 h. Each point represents the mean of 12 replicates evolved from each strain background.

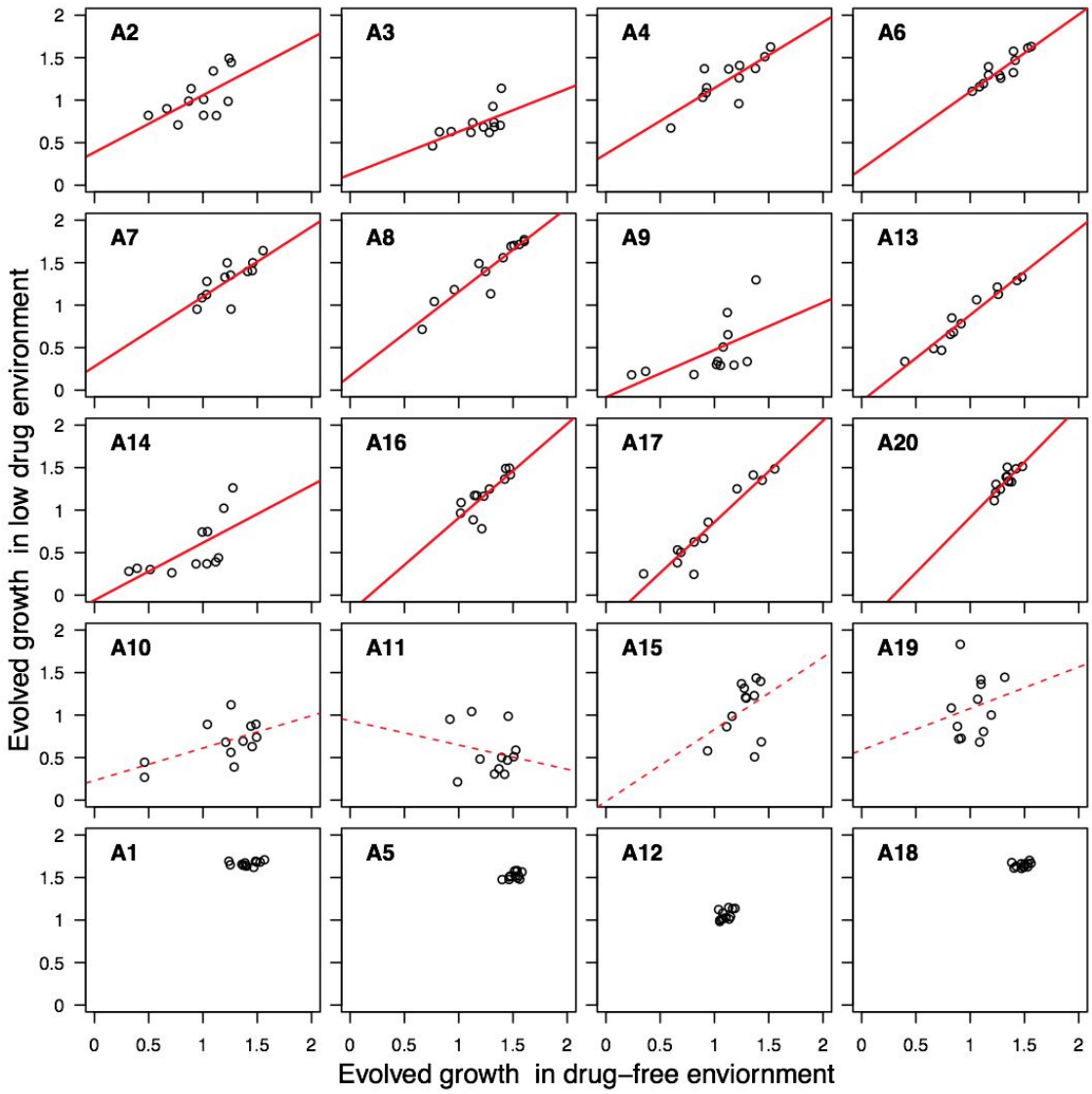

Figure S2. Evolved growth in a drug-free environment (YPD) is correlated with evolved growth in the evolutionary (low drug) environment in many strain backgrounds. Growth in both environments was measured as optical density at 24 h. Each point represents an independently evolved replicate from the same 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.

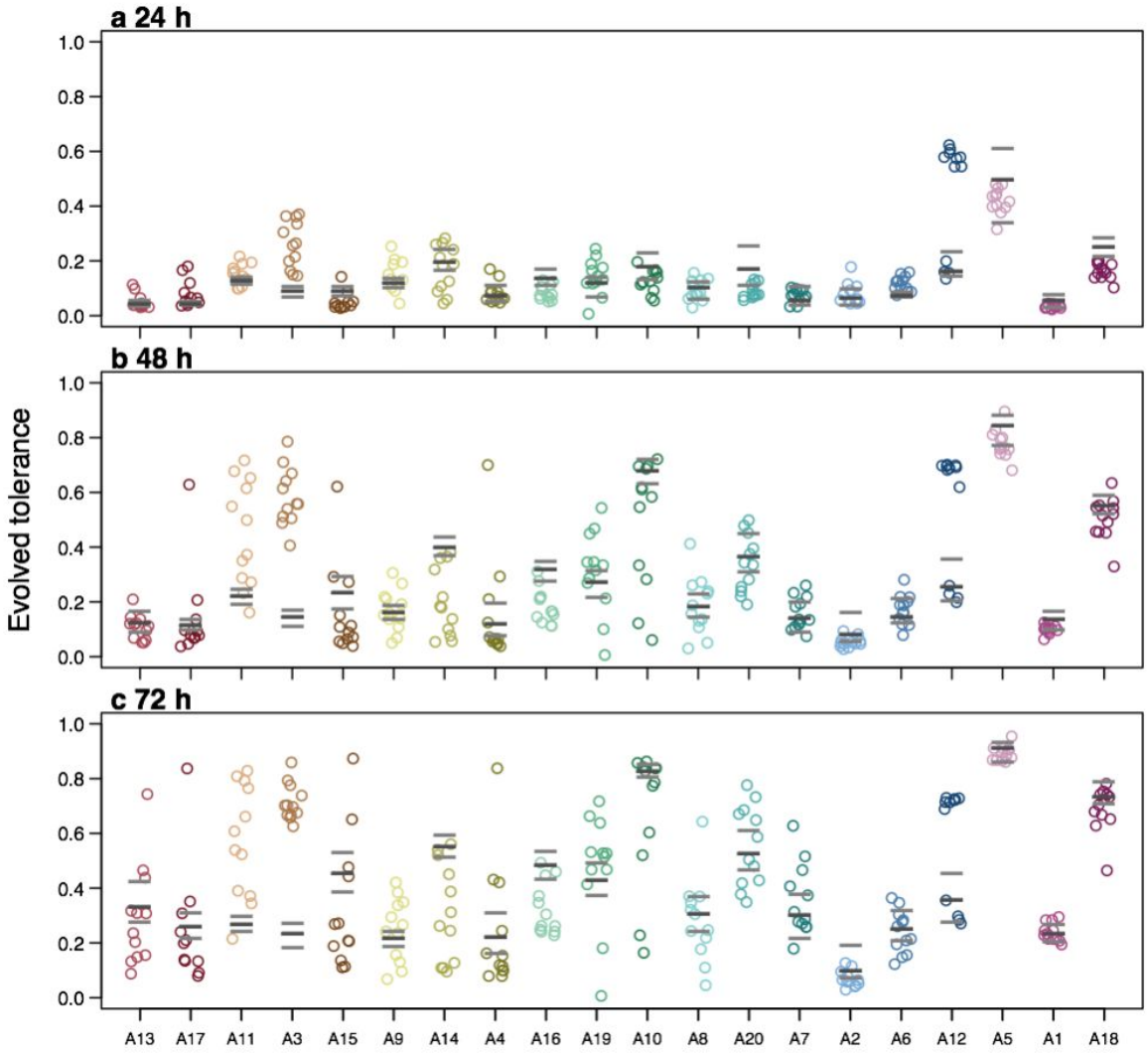

Figure S3. Tolerance of evolved strains measured at a) 24 h, b) 48 h, and c) 72 h. Tolerance was measured as the growth observed in supra-MIC levels of fluconazole (as appropriate to each replicate) normalized to the growth in the absence of drug. Strains are arranged on the x-axis (and coloured) by 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.

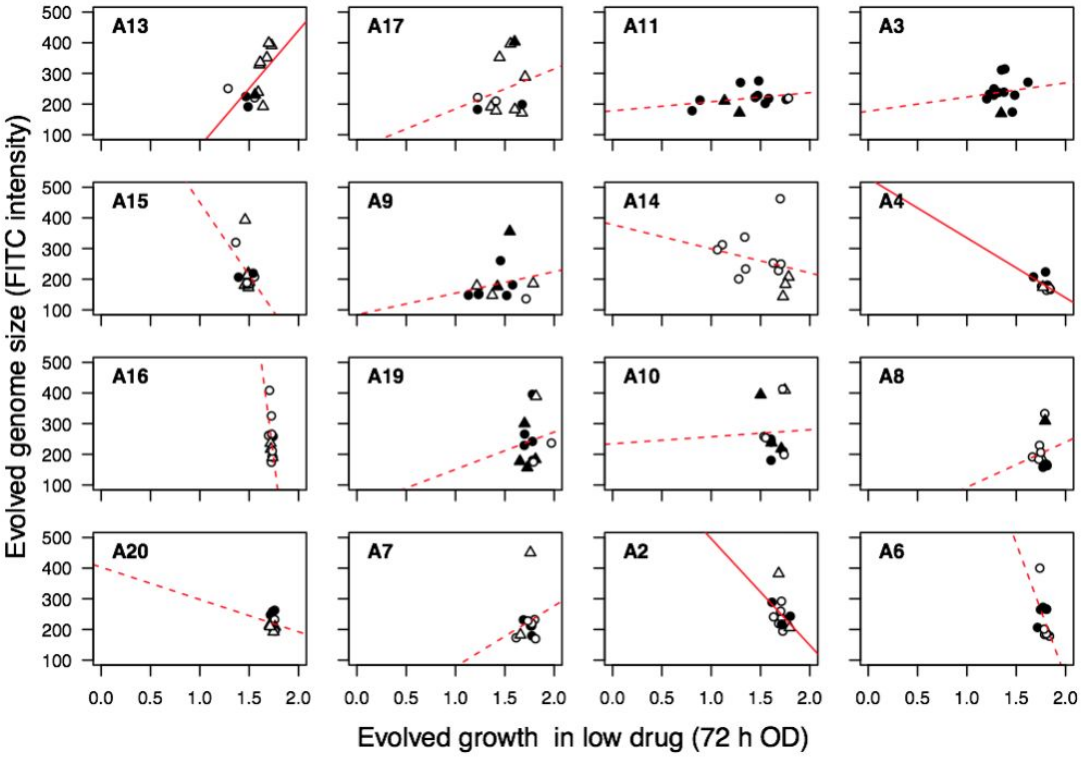

Figure S4. Evolved growth at 72 h and evolved genome size are not consistently correlated. Growth was measured as optical density after 72 hours in low drug. Evolved genome size indicates the most prominent G1 peak; when multiple G1 peaks were present this is indicated with a triangle symbol. Solid lines indicate a significant Spearman correlation test. Each point represents an independently evolved replicate line.

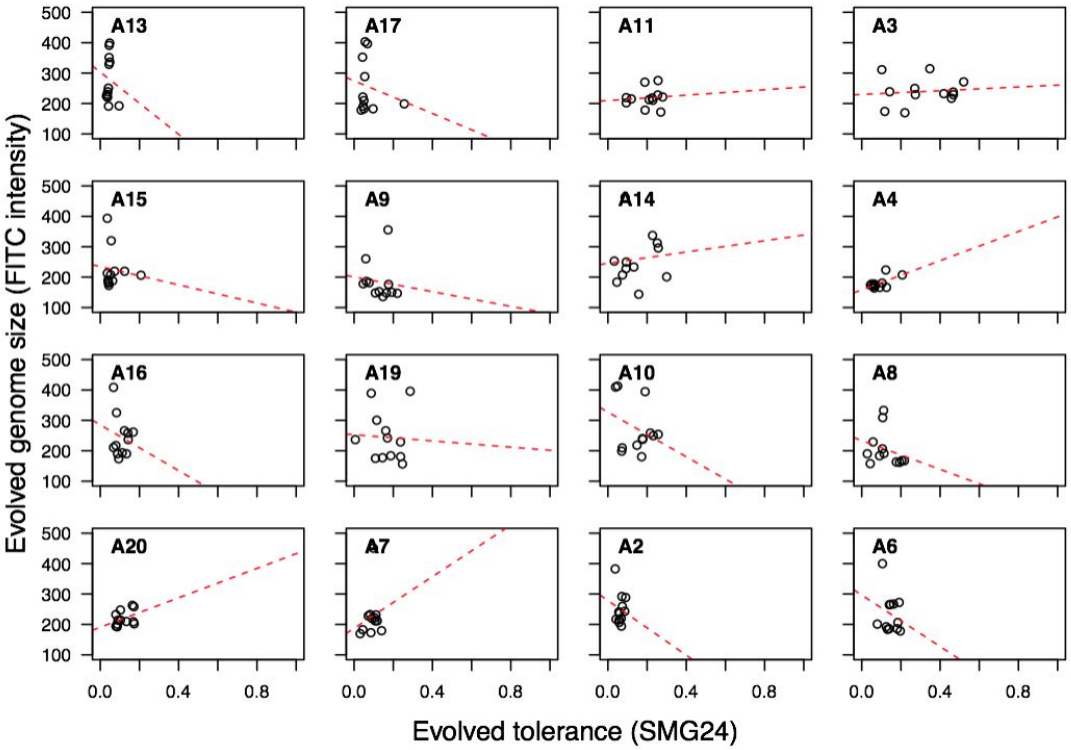

Figure S5. Evolved tolerance at 24 h and evolved genome size are only correlated in a single strain background. Each point represents an independently evolved replicate line. Tolerance was measured as SMG24. Evolved genome size represents the most prominent G1 peak; when multiple G1 peaks were present this is indicated with a triangle symbol. A solid line indicates a significant Spearman correlation test.

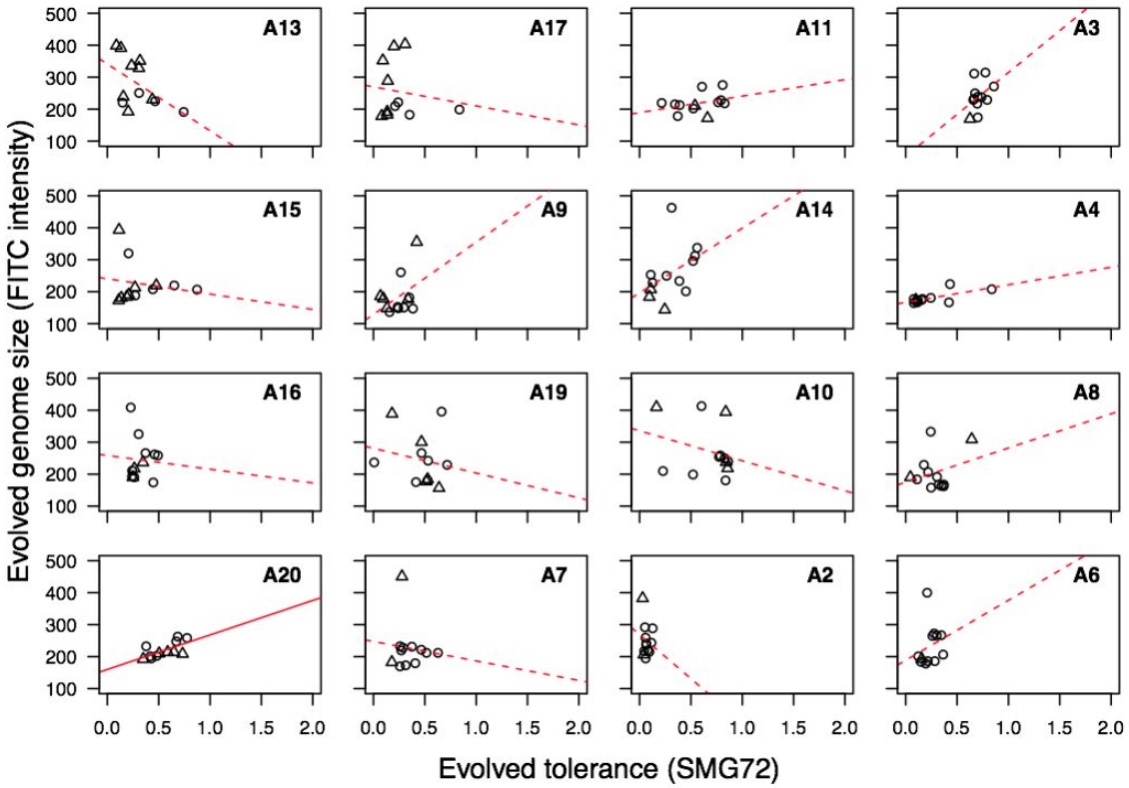

Figure S6. Evolved tolerance at 72 h and evolved genome size are only correlated in a single strain background. Each point represents an independently evolved replicate line. Tolerance was measured as SMG72. Evolved genome size represents the most prominent G1 peak; when multiple G1 peaks were present this is indicated with a triangle symbol. A solid line indicates a significant Spearman correlation test.