Evolution of drug-resistant and virulent small colonies in phenotypically diverse populations of the human fungal pathogen *Candida glabrata*

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Abstract

Antimicrobial resistance frequently carries a fitness cost to a pathogen, measured as a reduction in growth rate compared to the sensitive wild type, in the absence of antibiotics. Existing empirical evidence points to the following relationship between cost of resistance and virulence. If a resistant pathogen suffers a fitness cost in terms of reduced growth rate it commonly has lower virulence compared to the sensitive wild type. If this cost is absent so is the reduction in virulence. Here we show, using experimental evolution of drug resistance in the fungal human pathogen *Candida glabrata,* that reduced growth rate of resistant strains need not result in reduced virulence. Phenotypically heterogeneous populations were evolved in parallel containing highly-resistant sub-population small colony variants alongside sensitive sub-populations. Despite their low growth rate in the absence of an antifungal drug, the small colony variants did not suffer a marked alteration in virulence compared with the wild-type ancestral strain, or their co-isolated sensitive strains. This contrasts with classical theory that assumes growth rate to positively correlate with virulence. Our work thus highlights the complexity of the relationship between resistance, basic life-history traits and virulence.

Keywords
growth rate, virulence, drug resistance, fungal populations

Introduction

The rise of antimicrobial resistance (AMR) presents a major challenge to modern healthcare [1] but it is ultimately an ecological problem [2]. In natural environments, be it within or outside a host, susceptible and resistant microorganisms compete with each other [3]. While the use of antimicrobials promotes the growth of resistant strains or species [4], resistance frequently carries a fitness cost that arises when the antimicrobial is withdrawn [5-8]. The question we address here is this: how do virulence and resistance traits interact, costs of resistance in particular?

For a pathogenic microorganism, resistance costs are commonly measured as reductions in growth rate [5, 7, 9] which are often associated with reductions in virulence [8, 10, 11]. For example, resistant small colony variants isolated from otherwise susceptible populations [12] have lower growth rate than the suscpetible isolates they were found amongst in the absence of antibiotics but are also less virulent [13]. By contrast, resistant strains that do not suffer reduction in growth rates, either through “no-cost” or compensatory mutations, have been found not to have impaired virulence [8, 10, 14, 15]. Therefore, the existing empirical evidence suggests that the existence of virulent resistant or multi-resistant infections (as observed in [8]) is only possible when resistance costs are absent.

Here we challenge this reasoning by providing a counter example. While theory [16, 17] and within-species empirical studies [18, 19] argue that growth rate positively correlates with virulence, a between-species meta-analysis suggests that growth rate and virulence might not be positively correlated [20]. Thus, we hypothesise that virulence could be maintained following the evolution of costly resistance if a reduction in within-species growth rate does not lead to a reduction in virulence.

We deployed an experimental system using the human fungal pathogen *Candida glabrata*, ideally placed to test this hypothesis. In particular, the opportunistic pathogen *C. glabrata* undergoes rapid acquisition of resistance to the main classes of clinical antifungals [21, 22] and resistance can impart a growth fitness cost [11]. Crucially for testing our hypothesis, we recently observed attenuated virulence in a

fast-growing *C. glabrata* strain [23, 24] which would suggest the potential lack of a positive relationship between growth rate and virulence.

C. glabrata is a pathogen of clear clinical importance as it is the second most commonly isolated *Candida* species in bloodstream infections [25] with a greater than 50% death rate [26]. It is more closely related to the well-established model yeast *Saccharomyces cerevisiae* than to other species of the *Candida* genus [27]. As such, molecular, genetic and evolutionary techniques can readily be transferred from *S. cerevisiae* to *C. glabrata* making it an increasingly popular model organism to study infection [28-31].

After 14 seasons of transfer in post-MIC echinocandin (caspofungin) treatment, we found independent evolution of two *C. glabrata* heterogeneous populations consisting of diverse co-isolated colony morphologies with divergent resistance and growth properties. In both populations, a small colony variant phenotype was associated with a low growth rate and high resistance level. Interestingly, the small colony phenotype retained virulence in a wax moth larval infection model, comparable to the wild-type ancestor and co-isolated population colonies experiencing faster growth. These results suggest that virulence could be maintained in the presence of costly resistance that results in a reduced growth rate. In addition, we found no evidence of a correlation between *in vitro* growth rate and virulence across sub-population colony variants. Our work therefore questions the current understanding of the relationship between growth rate and virulence.

Methods

In vitro evolution of *C. glabrata* populations on a gradient of caspofungin concentrations

The reference *C. glabrata* strain ATCC 2001 [32] was used as the wild-type ancestor of all evolving replicate populations and was denoted as '2001WT'. Triplicate populations were evolved across a gradient of eight caspofungin concentrations of clinical relevance [33] and drug-free condition in a 96-well microtiter plate (Supplementary Figure 1a) in 10 mg ml^{-1} glucose Synthetic Complete (SC) medium (0.67% w/v yeast nitrogen base without amino acids and 0.079% w/v synthetic complete supplement mixture (Formedium)). Caspofungin concentrations in SC were

prepared from stock solution (5 mg ml^{-1}). Populations were serially transferred (1:30 dilution) every 24 hours to fresh media and drug conditions for 14 days. The 96-well plate was incubated at 30°C over 24 hours with shaking. OD (Optical Density) was read at 650 nm wavelength in a microtiter plate reader. The whole experiment was repeated on three separate 14-day periods (Experiments A, B and C).

OD data was blank-corrected and percentage relative growth at 24 hours, for each drug-treated population, was presented relative to the mean OD of the no drug-treated populations [34] (Supplementary Figure 2). We used the *lme4* package [35] with R version 3.4.3 [36] to conduct a linear mixed effects analysis of the fixed effects of day of the evolutionary experiment and caspofungin concentration on relative growth of *C. glabrata*. 'Population' was included as a nested random effect within 'Experiment' as each population was repeatedly measured across days of the evolutionary experiment. We obtained p-values from likelihood ratio tests.

Growth profiling of caspofungin-evolved endpoint colonies

All nine *C. glabrata* populations (Experiments A-C) that were evolved at 0.78, 1.37 and 2.40 µg/ml of caspofungin were revived from day 14 (Supplementary Figure 1b). We identified two distinct colony size variants hereby named SCV (Small Colony Variant) and RCV (Regular Colony Variant) in a single population from each of Experiment A and B but not C, and only at 0.78 µg/ml, shown in Supplementary Figure 3. Colony variants from Experiment A were growth-profiled in SC 1% w/v glucose media across replicate wells of a 96-well plate (N=4 wells x 3 separate day repeats = 12). OD was measured over 24 hours. Data from Experiment A are plotted in Figure 1a and b. Growth was measured in 4 replicate wells (N=4) of a 96-well plate for each of the SCV and RCV from Experiment B with data plotted in Supplementary Figure 4a and b.

Data was imported into MATLAB [37] to approximate intrinsic growth rate via logistic growth model fitting as described in [24], with exclusion of the lag phase parameter *L*. Relative growth rate and final growth yield were obtained by dividing through by the mean values of the 2001WT strain. We fitted a linear mixed effects model to relative growth rate and yield data from Experiment A and Experiment B using the *lme4* package [35] with R version 3.4.3 [36]. We included colony variant type as a fixed

factor and day of measurement as a random factor. A likelihood ratio test was used to test significance of the fixed factor.

Dose response profiling

We measured the caspofungin dose response profile over 24 hours for the co-isolated small and regular-sized colony variants from Experiments A and B and the wild-type ancestral strain (2001WT), to calculate IC50 (drug concentration causing 50% growth inhibition). Dose responses were set up in 96-well microtiter plates, using the same methods as described in the first season of the *in vitro* evolution assay (Supplementary Figure 1a). For colony variants that were resistant to concentrations up to 2.40 µg/ml, a two-fold dilution series from 64 µg/ml was used. Microtiter plates were incubated at 30^{o}C over 24 hours with orbital shaking and OD measurement. Dose responses were repeated three times independently for Experiment A (N = 3 wells x 3 repeats = 9 per caspofungin concentration) shown in Figure 1c and d. Dose responses were run once for Experiment B (N = 3 per caspofungin concentration) shown in Supplementary Figure 4c and d. The best-fit 4-parameter logistic dose response was plotted using R-package 'drc' [38], as described in [34] and used to estimate IC50.

Small colony phenotypes

Serial passaging

We tested stability of the randomly-selected SCV isolated from the single evolved population exhibiting colony diversity from each of Experiments A and B. A single overnight culture of each SCV was adjusted to 6.49 x 10^5 cells/ml, diluted 2-fold and serially passaged (1:30 dilution) across triplicate populations in 10 mg ml^{-1} glucose SC medium over 14 days. (Supplementary Figure 1d). The plate was incubated at 30°C with shaking at 180rpm and OD was measured during each 24-hour season. Growth rate and yield data from the final growth cycle are plotted in Figure 2 alongside 2001WT, with two-sample t-tests used to detect growth differences between 2001WT and passaged populations of each SCV. Caspofungin dose response was measured for a single clone cultured from each preserved endpoint (day 14) population (Supplementary Figure 1d), across triplicate wells for each caspofungin concentration. Endpoint passage population colony morphologies and

dose response are presented in Supplementary Figure 5 for a single population (representative of three) of the passaged SCV from each of Experiment A and B.

Characterisation of genomic targets

Genomic DNA was extracted from 2001WT and the single isolated SCV and RCV variants from each of Experiments A and B by mechanical cell lysis as described in [39]. Briefly, cells were grown overnight, centrifuged and mixed with Smash and Grab Solution (1% SDS, 2% Triton-X, 100 mM NaCl, 10 mM Tris pH 8.0), phenol-chloroform and acid-washed glass beads. The HS1 and HS2 regions of the *FKS1* and *FKS2* genes were amplified by PCR and Sanger-sequenced using primers previously described [40, 41]. Amplification of genes *CDC6, DOT6, MRPL11, SUI2* was performed using primers described for *C. glabrata* [11].

Competitive fitness assay between SCV and RCV (Experiment A)

To test competitive fitness of the stable SCV isolated from Experiment A against its co-isolated RCV, the two colony variants were competed across a set of mixed strain ratios in SC 1% glucose medium supplemented with 0.78 µg/ml caspofungin in wells of a 96-well plate (Supplementary Figure 6). Each strain was adjusted to 6.49 x 10^6 cells/ml prior to mixing and subsequent 2-fold dilution in SC media. Each mixed strain ratio was added to triplicate wells. The plate was incubated at 30°C with shaking at 180rpm. Competition mixes were plated on SC 1% glucose agar plates at the start (N=3) and end (N= 3 wells x 3 plates =9) of 24-hour growth, on which the colony morphotypes could clearly be distinguished by size. Relative fitness of the SCV was calculated as the ratio of the Malthusian growth parameters of the SCV and RCV [42]. A least-squares linear regression of relative fitness against initial SCV frequency was plotted and statistically analysed in Excel [43]. Two-tailed one-sample t-tests in R version 3.4.3 [36] tested significant deviations of relative fitness values from one.

Galleria mellonella survival assays

The 2001WT strain, regular colony, unpassaged and passaged small colony variants were tested for virulence in *G. mellonella* wax moth larvae. *C. glabrata* strains were grown in SC (2% glucose) media for 24 hours at 30ºC, cells were washed twice in PBS, counted on a haemocytometer and adjusted to 2.5 x 10^8 cells/ml in PBS with 20 µg/ml ampicillin. Groups of 20 wax moth larvae (UK Waxworms Ltd, Sheffield),

selected based on their weight (0.25-0.35g) and lack of visible melanisation, were injected with 10 µl spore suspension (2.5 x 10^6 CFU / larva) using a 50 µl Hamilton syringe into their last left pro-leg. Larvae were maintained at 37 ºC in the dark and monitored for survival. Three independent replicates of survival assays were performed on separate days (N=20 x 3 = 60 larvae in total per *C. glabrata* strain). A control group of 10 larvae injected with PBS and ampicillin were run alongside each independent replicate, and no deaths were seen. Mean survival time and log-rank comparisons of survival curves were calculated using OASIS 2 [44] for each of the three replicates. As routinely done with *G. mellonella* survival assays [45, 46], data from one of the replicates is presented in the main manuscript (Figure 3) while the data from the other two are presented in Supplementary Material (Supplementary Figures 7 and 8).

Testing for correlations between larval survival time and growth rate or yield

The mean relative growth rates or yields for each of the strains from Experiments A and B (reported in Figures 1a and b, 2, and Supplementary Figure 4a and b) were plotted against their virulence calculated as larval survival time from either Figure 3 (first replicate survival study), Supplementary Figure 7 (second replicate survival study) or Supplementary Figure 8 (third replicate survival study); this generated Figure 4, Supplementary Figure 10a and Supplementary Figure 11a, respectively. Note, as growth trait replicate measurements were not directly paired with larval survival time measurements, their mean values were plotted with standard error bars representing the variability in estimation. Bootstrapping was performed on data in Figure 4, Supplementary Figure 10a and Supplementary Figure 11a both for Linear and Deming regressions and also for Pearson and Spearman correlations (using MATLAB's Statistics and Machine Learning Toolbox) [37]. The resulting statistics are reported in Supplementary Figures 9a and b and Supplementary Figures 10-11b and c.

Results

Rapid evolution of sub-population phenotypic diversity with antifungal caspofungin treatment

We measured growth adaptations of populations of the reference *C. glabrata* strain ATCC 2001 (denoted 2001WT), serially transferred over 14 days and evolving across a gradient of 8 antifungal caspofungin concentrations (see Methods). Growth across *C. glabrata* populations, relative to no-drug treated controls, significantly increased over time (effect of day: likelihood ratio test: $\chi^2(2) = 52.297$, p =4.404e-12) (Supplementary Figure 2). Growth was also significantly influenced by caspofungin concentration (likelihood ratio test: χ^2 (1) = 154.6, p < 2.2e-16). The greatest relative population growth increase from 3.0 +/- 2.5 (SE) % on day 1, to 88.8 +/- 7.0 (SE) % on day 14 occurred at 0.78 µg/ml of caspofungin.

We revived triplicate endpoint (day 14) populations that were evolved at the three highest caspofungin concentrations (0.78, 1.37 and 2.40 µg/ml), to phenotype colony morphologies (Supplementary Figure 1b). We identified colony size variation in a single population from Experiment A and a single population from Experiment B, which had both been evolved during treatment in 0.78 µg/ml caspofungin. Two size variants were observed in each of these populations: a small colony variant (SCV) and a regular colony variant (RCV) (Supplementary Figure 3). We did not observe the SCV phenotype in any populations from Experiment C. The small colony morphology and lower growth rate of the isolated SCV resembled the respiratory-deficient petite phenotype previously observed for *C. glabrata* [11], however our SCV strains were able to grow on a non-fermentable carbon source, indicating respiratory function (Supplementary Figure 3).

We measured growth rates and final population densities (yield) of the SCV and RCV strains, relative to strain 2001WT in the absence of caspofungin. Relative growth rate significantly varied with colony type (Experiment A (Figure 1a): $\chi^2(2) = 73.376$, p < 2.2e-16; Experiment B (Supplementary Figure 4a): $\chi^2(2) = 69.543$, p = 7.926e-16). No significant differences were found between 2001WT and RCV, but growth rate of SCV was significantly lower than both 2001WT and RCV.

Relative growth yield significantly varied with colony type (Experiment A (Figure 1b): $\chi^2(2) = 21.454$, p = 2.195e-05; Experiment B (Supplementary Figure 4b): $\chi^2(2) =$ 34.644, p = 3e-08). Growth yield of the SCV was significantly greater than both

2001WT and the RCV. In Experiment B only, yield of the RCV was significantly lower than 2001WT.

We next measured the caspofungin dose response of the variants from Experiments A and B that were growth profiled. Caspofungin susceptibility profiles of the two colony variants were highly divergent. From Experiment A, the RCV (Reg_Col) had an IC50 of 0.26 +/- 0.007 µg/ml that was slightly lower (marginally significantly) than the 2001WT strain IC50 (0.32 +/- 0.0025 µg/ml; p = 0.0454) (Figure 1c). The co-isolated SCV (Small_Col) however was not susceptible to caspofungin concentrations up to 2.4 µg/ml (Figure 1d) and had an IC50 of 2.61 +/- 0.136 µg/ml, approximately 10-fold greater than the IC50 of the co-isolated regular colony variant and 8.2-fold greater than the 2001WT strain.

In Experiment B, the RCV (Reg_Col) had an IC50 of 0.83 +/- 0.096 µg/ml, significantly greater than the 2001WT strain (p = 0.0011; Supplementary Figure 4c). The co-isolated SCV (Small_Col) was not sensitive to caspofungin concentrations up to 2.4 µg/ml (Supplementary Figure 4d) and had an IC50 of 4.71 +/- 0.236 µg/ml, 5.7-fold greater than the regular co-isolated colony variant and 14.7-fold greater than the 2001WT strain.

Divergent phenotypic stability of independently-evolved SCVs, in the absence of drug

We investigated stability of the resistant small colony variants, individually isolated from the single population from each of Experiment A and B. For this we serially passaged three replicate populations seeded from an individual culture of each SCV (from Experiment A and B), over 14 days in the absence of caspofungin. For Experiment A, the small colony morphology and resistance level showed no reversion via appearance of regular-sized colonies after 14 days. The ‘stable’ small colony phenotype from Experiment A maintained a significantly lower relative growth rate than the 2001WT strain on the 14^{th} day of transfer in the absence of caspofungin ($t(13) = 21.952$, $p = 1.169e-11$) (Figure 2a). However, after passage relative growth yield did not significantly differ from the wild-type ($t(13) = 1.7375$, $p = 0.1059$) (Figure 2b). The level of resistance displayed by the small colony variant was maintained, with no growth inhibition between 0 – 2.4 µg/ml of caspofungin (Supplementary

Figure 5: Experiment A). The IC50 values for a single colony of each passaged population of the SCV from Experiment A (experimental setup as shown in Supplementary Figure 1d) were as follows: 2.40 +/- 0.146 µg/ml (population 1), 2.33 +/- 0.678 µg/ml (population 2) and 2.70 +/- 0.480 µg/ml (population 3)). This indicated an underlying stable resistance mechanism and a lack of compensatory fitness improvement.

In contrast, the resistant small colony phenotype from Experiment B was homogeneously reversed in all three passaged replicate populations after 14 days. The 'unstable' small colony phenotype from Experiment B had lost its lower relative growth rate by the 14^{th} transfer day and was not significantly different from 2001WT ($t(13) = 1.2265$, $p = 0.2418$) (Figure 2c). This small colony variant after passage showed a significant 1.2-fold lower relative growth yield than the wild type ($t(13) = 3.5315$, $p = 0.003685$) (Figure 2d).The resistance level of the passaged populations decreased but sensitivity levels (IC50 values) were not completely restored to wild-type levels: in two populations the IC50 values were higher than 2001WT (0.49 +/- 0.023 µg/ml; $p = 0.000336$ (Supplementary Fig. 5: Experiment B- population 1) and 0.44 +/- 0.019 µg/ml; $p = 0.00463$ (population 2)) but were comparable in the third population (0.34 +/- 0.055 µg/ml; $p = 0.797$ (population 3)). Despite independent evolution of the small colony phenotype in separate populations during caspofungin treatment, the findings indicate that the SCV isolated from Experiment B evolved a different but transient mechanism of resistance compared with the SCV from Experiment A.

We tested whether resistance seen in the small colony phenotype from Experiment A and B correlated with genetic differences in hotspot regions of the *FKS* genes, which are common targets associated with caspofungin resistance [11, 40]. Sequenced regions were identical in all strains with no nucleotide changes detected. Furthermore, we identified no nucleotide differences between strains in an additional set of genetic targets (*CDC6, DOT6, MRPL11, SUI2)* putatively associated with development of caspofungin resistance [11].

We then tested competitive fitness of the isolated SCV against the RCV from the evolved population in Experiment A, over 5 different initial frequencies of the SCV in

the presence of caspofungin (0.78 μg/ml). Relative fitness of the SCV was significantly greater than one for all initial frequencies, apart from the highest starting SCV frequency (0.95) when there was no significant difference (Supplementary Figure 6). Relative fitness of the SCV was significantly negative frequency-dependent (least-squares linear regression: slope = -4.8990, t = -7.554, df = 42, p = 2.37e-09). These results lead to a longer-term prediction that a low-frequency of an RCV could co-exist with a high frequency of an SCV, however the underlying mechanisms of this dynamic were not explored here.

Independently-evolved drug-resistant SCVs are not attenuated in virulence

We tested virulence of 2001WT, small colony variants (Experiments A and B) and co-isolated regular colony variants in the wax moth model *Galleria mellonella*. The 'stable' small colony variant from Experiment A was virulent in *G. mellonella* both before and after passaging without caspofungin (Figure 3a). Mean larval survival times were 2.61 ± 0.56 days and 1.93 ± 0.49 days respectively, and we found no significant differences from 2001WT (2.01 ± 0.44 days; log-rank test p-values = 0.3481 (ExA-S); 0.9834 (ExA-P)) or the co-isolated regular colony variant (2.15 ± 0.51 days; p = 0.438 (ExA-S); 0.9483 (ExA-P)). For the 'unstable' small colony variant from Experiment B we found no significant difference in *G. mellonella* mean survival times when comparing states before (1.54 ± 0.32 days) and after (1.14 ± 0.18 days) loss of the phenotype (p = 0.2393) (Figure 3b). No significant differences in mean larval survival time occurred between 2001WT and either ExB-S (p = 0.5338) or ExB-P (p = 0.08), nor between ExB-R (1.25 ± 0.31 days) and either ExB-S (p = 0.2825) or ExB-P (p = 0.8389). These findings were consistent with two additional independent replicate survival studies conducted on separate days (Supplementary Figures 7 and 8).

Bootstrapping analysis tested for, but found no evidence of, correlations between larval survival time (measure of virulence) and either relative growth rates or yields in 3 replicate survival studies (Figure 4, Supplementary Figures 9, 10 and 11). Further *G. mellonella* infection studies involving larger sample sizes of drug-evolved clones would be needed to establish evidence for an absence of correlation.

Discussion

Our work demonstrates that drug-resistance cost in the form of reduced growth rate, does not necessarily lead to a marked reduction in virulence. This challenges the current understanding of the resistance-virulence relationship [8]. Using a clinical isolate of a deadly human pathogen *C. glabrata* [25, 26] we conducted *in vitro* evolutionary studies in the presence of an antifungal drug and repeatedly evolved a resistant small colony variant (Figure 1 and Supplementary Figure 4). Despite having a significantly reduced growth rate compared to the susceptible wild-type, the small colony variants did not suffer a marked alteration in virulence when tested in *G. mellonella*, a well-established model host for detecting virulence differences in *C. glabrata* strains [23]. The isolated small colony variants represented a subpopulation of highly resistant individuals, amongst susceptible sub-populations within a drug-treated population – a hallmark of heteroresistance [12]. Increasingly observed amongst fungal [10, 47] and bacterial [12] pathogens, heteroresistance presents challenges for detection and antibiotic susceptibility testing [12]. This can lead to persistent and recurrent infections [48-50] and missed detection of resistant subpopulations [51].

At the first glance, the small colony morphology and lower growth rate of our isolated small colony variants resembles the well-known *C. glabrata* petite phenotype [11, 52, 53]. *C. glabrata* is known to rapidly evolve resistance to caspofungin [11], a front-line therapeutic for *Candida* infections, also used in our study. During patient treatment with caspofungin for recurring bloodstream candidemia, resistant isolates including a small colony phenotype were recovered [11]. These small colony isolates were found to be respiratory-deficient having lost mitochondrial function and were termed petite mutants. The petite phenotype was first observed in the yeast *Saccharomyces cerevisiae* [54] and more recently identified in *C. glabrata* azole-resistant isolates [52, 53]. However, unlike previously identified caspofungin-resistant petite mutants [11] our small colony variants do not suffer a reduction in virulence (Figure 3) and can grow on media containing glycerol as a non-fermentative carbon source, indicating respiratory function (Supplementary Figure 3). This indicates potential presence of a different resistance mechanism that co-occurs with virulence, not previously observed for the cidal (cell-killing) echinocandin drug class [11]. In line with our findings, a prior study that isolated a *C. glabrata* azole-resistant petite during clinical infection identified that it had enhanced virulence but reduced *in vitro* fitness in the

absence of the drug, compared to the sensitive co-isolated type [55]. Thus we argue that detection of reduced *in vitro* growth rate of a sub-population clone [11] is not a reliable indicator of virulence.

Our study also indicates that small colony resistance in response to the front-line antifungals can be both reversible and non-reversible (Supplementary Figure 5) when the drug is removed. So far, studies on the small colony phenotype have shown reversibility of growth rate and either reversibility or non-reversibility of resistance [56]. For example, petite *C. glabrata* mutants result from stable genetic alteration of mitochondrial DNA and show upregulation of drug efflux transporters when evolved with azoles [52, 53, 57]**.** Stability over passaging in the absence of drug, for the first study to isolate *C. glabrata* petite mutants during echinocandin treatment, was not investigated [11]. In contrast, heteroresistance has consistently proven reversible for both clinical and environmental isolates of *Cryptococcus neoformans* exposed to most commonly used antifungals [58]. Other studies have shown that resistance achieved through induction is reversible, whilst resistance achieved through selection is non-reversible in a range of *Candida* species [59]. Here we show that different resistance mechanisms could be acting in parallel giving rise to either reversibility or non-reversibility. Recent studies of bacterial heteroresistance describe stable heteroresistance to be associated with minimal fitness costs, whereas unstable heteroresistance has been associated with larger fitness costs leading to compensatory changes that often reverse resistance [60]. In contrast, we find low growth rates for both our reversible and non-reversible small colony variants. Moreover, we show that the resistant small colony phenotype can be stable with passaging in the absence of antibiotics, without compensatory mechanisms that restore growth rate as was previously described as a necessity for stability [56]. This highlights the need for future studies to investigate repeatability of heteroresistance development and stability across multiple parallel populations. This will lead to better predictability of evolutionary pathways to resistance development in pathogen populations [61].

Classical theory assumes that growth rate positively correlates with pathogen fitness and virulence [16, 62-64] due to debilitation of the host [65]. Despite some empirical support of this classical assumption [19, 66], recent meta-analysis across 61 human

pathogens found that growth rate was negatively correlated with virulence [20]. Moreover, comparisons of *C. glabrata* clinical strains found that a fast grower was less virulent than slower growers [23] with fast growth also being constrained by a low growth yield [24]. Here, we found no evidence of correlation between virulence and either growth rate or growth yield.

Our study further highlights the complex nature of the relationship between pathogen growth rate and virulence. In particular, existing empirical studies [20, 23, 66, 67] including ours conducted here, compare virulence between pathogenic strains with undefined genetic differences. As a result, observed virulence variations cannot be mechanistically linked to differences in growth traits. In general, this could be due to confounding effects such as differences in host responses to genetically diverse pathogens [68] or undefined interactions between pathogen strains [69].

Here we argue that in order to understand the effect of resistance costs on the evolution of virulence we need to develop an in-depth, mechanistic understanding of the relationship between growth rate and virulence.

Data accessibility

Additional details of methods and results including Supplementary Figures are available in the Electronic Supplementary Material. Raw data are included in an Excel file.

Authors' contributions

S.J.N.D. and I.G. designed the study. S.J.N.D. and S.B. performed the experiments. S.J.N.D. and R.E.B completed data presentation, analysis and statistics. S.J.N.D., R.E.B, S.B. and I.G. wrote the manuscript.

Competing interests

We declare that we have no competing interests

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Figure legends

Figure 1. Sub-population colony diversity in growth fitness and drug susceptibility in Experiment A. Data are shown for the isolated regular-sized colony variant and small colony variant from the single population in which colony diversity was identified. Reg_Col (RCV) = regular-sized colony variant; Small_Col (SCV) = small colony variant); wild-type ancestral strain = 2001WT. In (a) and (b), growth values are calculated relative to average values of 2001WT, where a value of 1.0 signifies no change relative to ancestor. N = 12 measurements per colony variant (measurement in four individual wells of a microtiter plate, repeated separately on three days). Different symbol shapes (separated by horizontal noise on the x-axis) represent measurements from separate days. Black points and error bars show overall means and standard errors. Average relative growth rates (+/- SE): Reg_Col: 0.91 +/- 0.07; Small_Col: 0.09 +/- 0.05. Average relative growth yields (+/- SE): Reg_Col: 0.99 +/- 0.01; Small_Col: 1.13 +/- 0.03. Dose response plots (c) and (d) show final OD of drug-treated populations as a percentage of average growth of the no-drug treated populations. The same dataset for the ancestral strain (2001WT) is plotted in (c) and (d). N = 9 measurements per colony variant for each drug concentration (measurement in three individual wells of a microtiter plate, repeated separately on three days). Different symbol shapes represent measurements from separate days. Lines represent best fit of the logistic dose response model. Model-predicted IC50 values +/- SE of the estimated value are shown for each dose response.

Figure 2. Final relative growth fitness following passaging without caspofungin of the single isolated small colony variant from Experiment A and Experiment B. Plots (a) and (b) show data from the small colony variant isolated from Experiment A; plots (c) and (d) represent the small colony variant isolated from Experiment B. The wild-type ancestral strain (2001WT) data is as presented in Figures 1a and b (N = 12) as this strain was not passaged. N = 3 per small colony variant (different shape symbol per replicate), representing a single endpoint measurement from each of three passaged populations seeded from a single culture of the small colony variant. Growth values are calculated relative to the average values of 2001WT, where a value of 1.0 signifies no change relative to the ancestor. Experiment A: average

relative growth rate of SCV (+/- SE) = 0.05 +/- 0.04; average relative growth yield of SCV (+/- SE) = 1.09 +/- 0.01. Experiment B: average relative growth rate of SCV (+/- SE) = 1.06 +/- 0.09; average relative growth yield of SCV (+/- SE) = 0.81 +/- 0.05. Black points and error bars represent mean and standard error.

Figure 3. Virulence of *C. glabrata* wild-type ancestral, small and regular colony size variants in *G. mellonella* larvae. Survival of groups of 20 *G. mellonella* wax moth larvae injected with 2.5 x 10^6 CFU/larva per strain over 7 days incubation at 37°C. These data represent one independent replicate of the survival analysis; the second and third replicates are presented in Supplementary Figures 7 and 8. (a) WT = 2001WT ancestral strain, ExA-R = Experiment A regular colony variant, ExA-S = Experiment A small colony variant, ExA-P = Experiment A passaged small colony variant. (b) ExB-S = Experiment B small colony variant, ExB-R = Experiment B regular colony variant, ExB-P = Experiment B passaged small colony variant (revertant). No significant differences in survival in log-rank tests were found across Experiment A ($p \geq 0.3481$) or Experiment B strains ($p \geq 0.08$).

Figure 4. Growth rate, growth yield and virulence correlations. Growth traits are plotted for all strains from Experiments A and B, including regular colony variants and small colony variants before and after passaging. Data is combined from Figures 1a, b, 2, 3 and Supplementary Figure 4a and b. Plotted points represent mean values +/- SE. Growth rate and yield are plotted relative to the wild-type ancestral strain (2001WT). Strains are labelled as - Experiment A strains: ExA-R (regular colony variant); ExA-S (small colony variant); ExA-P (passaged “stable” small colony). Experiment B strains: ExB-R (regular colony variant); ExB-S (small colony variant); ExB-P (passaged “unstable” small colony). Bootstrapping was performed for both Linear and Deming regressions, in addition to both Pearson and Spearman but we found no evidence of correlations between virulence and either relative growth rate (Supplementary Figure 9a) or relative growth yield (Supplementary Figure 9b).

Figure 1

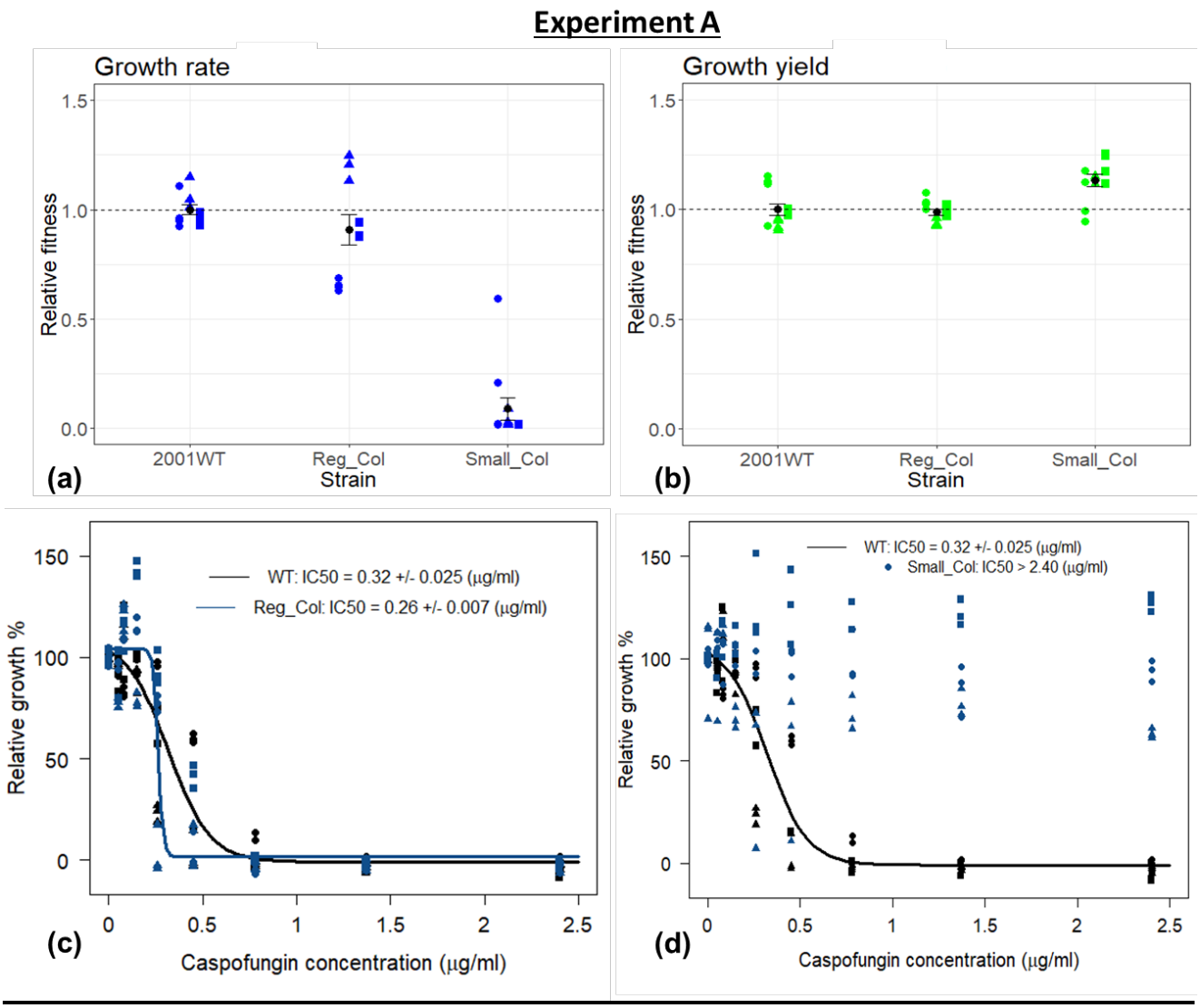

Experiment A
Growth rate
Relative fitness
1.5
1.0
0.5
0.0
2001WT
Reg_Col
Small_Col
Strain
(a)
Growth yield
Relative fitness
1.5
1.0
0.5
0.0
2001WT
Reg_Col
Small_Col
Strain
(b)
Relative growth %
150
100
50
0
WT: IC50 = 0.32 +/- 0.025 (µg/ml)
Reg_Col: IC50 = 0.26 +/- 0.007 (µg/ml)
0
0.5
1
1.5
2
2.5
Caspofungin concentration (µg/ml)
(c)
Relative growth %
150
100
50
0
WT: IC50 = 0.32 +/- 0.025 (µg/ml)
Small_Col: IC50 > 2.40 (µg/ml)
0
0.5
1
1.5
2
2.5
Caspofungin concentration (µg/ml)
(d)

Figure 2

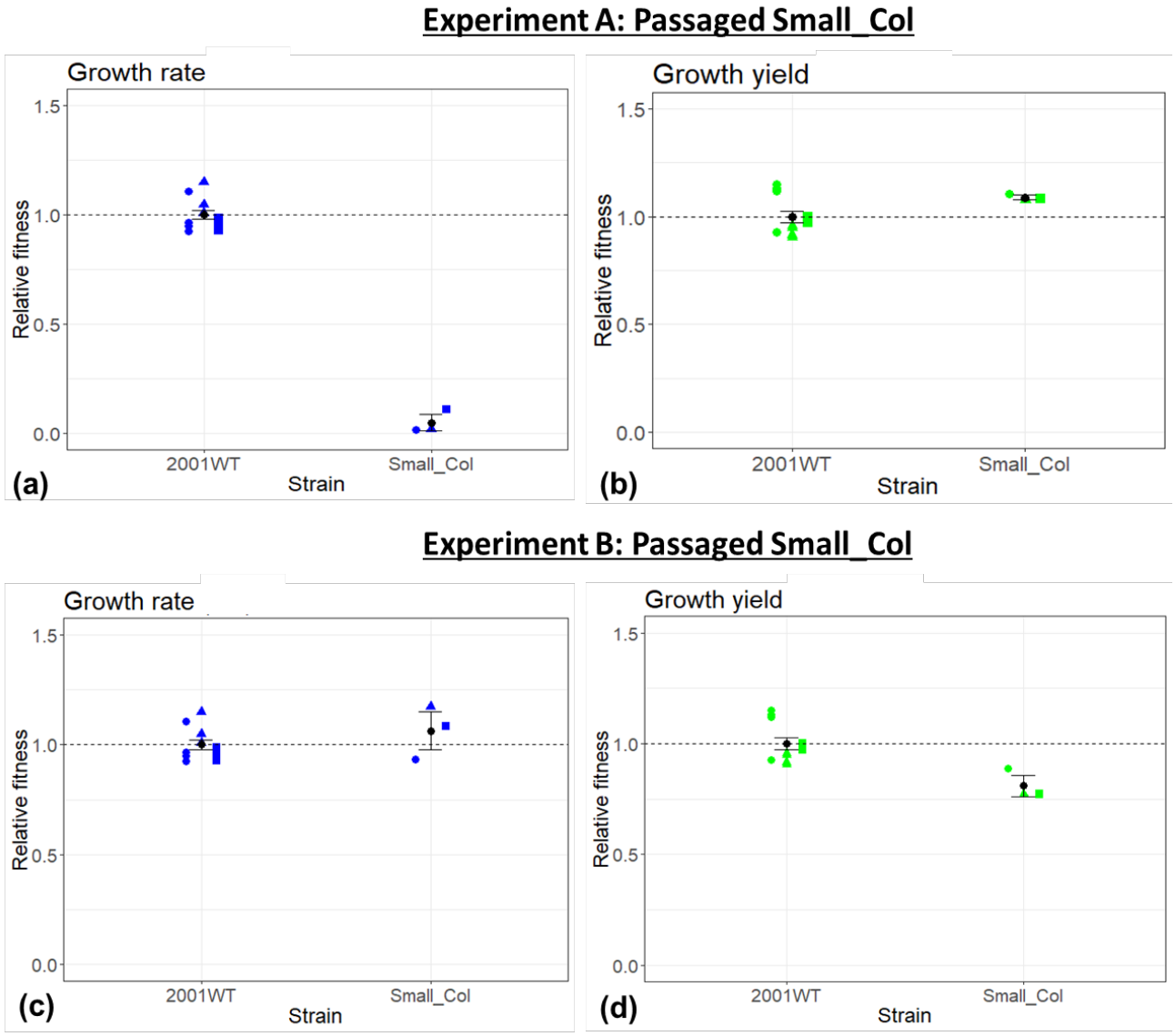

Experiment A: Passaged Small_Col
Growth rate
Growth yield
Relative fitness
1.5
1.0
0.5
0.0
2001WT
Small_Col
Strain
(a)
(b)
Experiment B: Passaged Small_Col
(c)
(d)

Figure 3

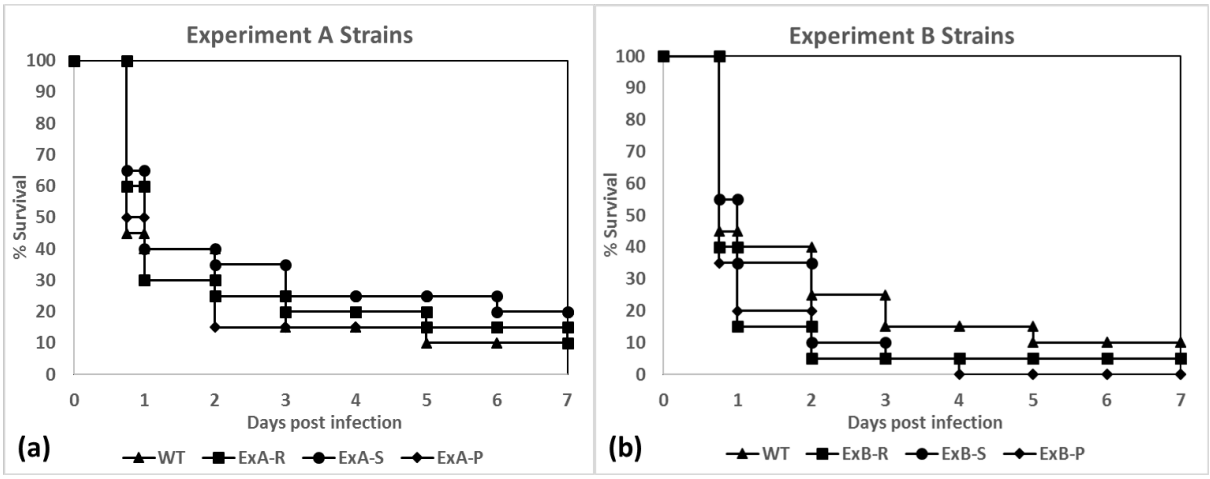
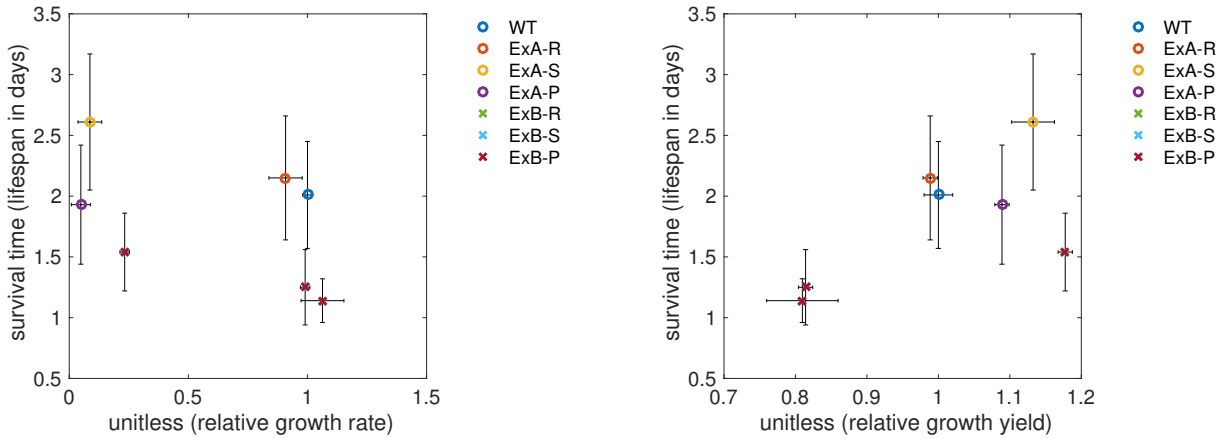


Figure 4



Electronic Supplementary Material

Evolution of drug-resistant and virulent small colonies in phenotypically diverse populations of the human fungal pathogen *Candida glabrata*

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Keywords: growth rate, virulence, drug resistance, fungal populations

SUPPLEMENTAL METHODS

In vitro evolution of *C. glabrata* populations on a gradient of caspofungin concentrations

The reference *C. glabrata* strain ATCC 2001 [1] was used as the wild-type ancestor of all evolving replicate populations and was denoted as '2001WT'. 2001WT was pre-grown overnight in YPD (Yeast Peptone Dextrose: 2% w/v bacteriological peptone, 1% w/v yeast extract, 2% w/v glucose) medium, cells were washed in PBS (phosphate-buffered saline) and re-suspended in SC medium (10 mg ml^{-1} glucose, 0.67% w/v yeast nitrogen base without amino acids and 0.079% w/v synthetic complete supplement mixture (Formedium)). Triplicate populations were evolved across a gradient of eight caspofungin concentrations (0.05, 0.08, 0.15, 0.26, 0.45, 0.78, 1.37, 2.40 µg/ml) of clinical relevance [2] and drug-free condition in a 96-well microtiter plate (experimental design shown in Supplementary Figure 1). Initial cell density per well was approximately 3.25 x 10^6 cells/ml. The 96-well plate was sealed with aerated transparent film and incubated at 30^{o}C over 24 hours with orbital shaking at amplitude 4mm. OD (Optical Density) was read at 650 nm wavelength in a Tecan M200 microtiter plate reader.

We used the *lme4* package [3] with R version 3.4.3 [4] to conduct a linear mixed effects analysis of the fixed effects of day of the evolutionary experiment and caspofungin concentration on relative growth of *C. glabrata*. 'Population' was included as a nested random effect within 'Experiment' as each population was repeatedly measured across days of the evolutionary experiment. We obtained p-values from likelihood ratio tests comparing the full model with alternative models with the individually-removed interaction term or individual fixed effects.

Growth profiling of caspofungin-evolved endpoint colonies

All nine *C. glabrata* populations (Experiments A-C) that were evolved at 0.78 µg/ml of caspofungin were revived from day 14. This was done by streaking out frozen day 14 populations on 10 mg ml-1 glucose SC agar and CHROMagar plates (BD Biosciences, Oxford, UK) (experimental design shown in Supplementary Figure 1). We identified two distinct colony size variants hereby named SCV (Small Colony

Variant) and RCV (Regular Colony Variant) in a single population from each of Experiment A and B but not C and only at 0.78 µg/ml, shown in Supplementary Figure 3. The 2001WT strain was growth profiled as a reference alongside a single SCV and RCV from the two independent populations (one from Experiment A and the other from Experiment B).

Relative growth rate and final growth yield were obtained by dividing through by the mean values of the 2001WT strain. We fitted a linear mixed effects model to relative growth rate and yield data from Experiment A and Experiment B using the *lme4* package [3] with R version 3.4.3 [4]. We included colony variant type as a fixed factor and day of measurement as a random factor. A likelihood ratio test was used to test significance of the fixed factor, by comparison of the full model with an intercept-only model. We reported significant between-colony variant differences when the 95% confidence interval for the difference in mean growth rate or yield did not span zero.

Dose response profiling

Dose responses were set up in 96-well microtiter plates, using the same methods as described in the first season of the *in vitro* evolution assay (Supplementary Figure 1a). Initial cell density per well was approximately 3.25×10^6 cells/ml.

Small colony phenotypes

Serial passaging

We tested stability of the randomly-selected SCV isolated from the single evolved population exhibiting colony diversity from each of Experiments A and B. A single overnight culture of each SCV was adjusted to 3.25×10^5 cells/ml and serially passaged (1:30 dilution) in triplicate populations in 10 mg ml^{-1} glucose SC medium over 14 days. Populations were serially diluted and plated on SC agar every 2 days to test for changes in colony phenotype, a sign of compensatory fitness change in resistant mutants [5].

Characterisation of genomic targets

The HS1 and HS2 regions of the *FKS1* and *FKS2* genes were amplified by PCR and Sanger-sequenced using primers previously described [6, 7]. Amplification of genes *CDC6, DOT6, MRPL11, SUI2* was performed using primers described for *C. glabrata* [8]. All PCR reactions contained: 25 µl GoTaq, 2 µl of forward primer (20 µM), 2 µl of reverse primer (20 µM), 1ul of DNA template and 20 µl of nuclease-free water. The PCR programme was run as follows: DNA denaturation- 95^{o}C for 2 minutes; 35 cycles: denaturation- 94^{o}C for 30 seconds, annealing for 45 seconds with adjusted temperature for each gene target, extension- 72^{o}C for 1 minute; final extension- 72^{o}C for 5 minutes. Nucleotide and amino acid sequences were aligned using MEGA software [9]. *FKS1* and *FKS2* hotspot 1 and 2 gene targets were sequenced in both the forward and reverse directions; other gene targets were sequenced just in the forward direction.

Competitive fitness assay between SCV and RCV (Experiment A)

To test competitive fitness of the stable SCV isolated from Experiment A against its co-isolated RCV, the two colony variants were competed across a set of approximate starting frequencies of the SCV (0.1, 0.3, 0.5, 0.7 and 0.9). A least-squares linear regression of relative fitness against initial SCV frequency was plotted. Coexistence between the SCV and RCV was predicted for the SCV frequency when relative fitness was equal to one. The significance of the regression slope was calculated in Excel [10]. Significance of relative fitness values above or below one were calculated by two-tailed one-sample t-tests in R version 3.4.3 [4]. Data are presented in Supplementary Figure 6.

SUPPLEMENTAL RESULTS

Divergent phenotypic stability of independently-evolved SCVs, in the absence of drug

We then tested competitive fitness of the isolated SCV against the RCV from the evolved population in Experiment A, over 5 different initial frequencies of the SCV in the presence of caspofungin (0.78 µg/ml). Relative fitness of the SCV was

significantly greater than one for all initial frequencies, apart from the highest starting SCV frequency (0.95) when there was no significant difference (Supplementary Figure 6). Relative fitness of the SCV was significantly negative frequency-dependent (least-squares linear regression: slope = -4.8990, t = -7.554, df = 42, p = 2.37e-09). The SCV had a greater fitness than the RCV when present at an initial frequency between 0.1 and 0.8 (relative fitness of SCV significantly greater than one). Above an initial SCV frequency of 0.85 (where the regression line intersected with SCV relative fitness of one), fitness of the SCV and RCV did not significantly differ.

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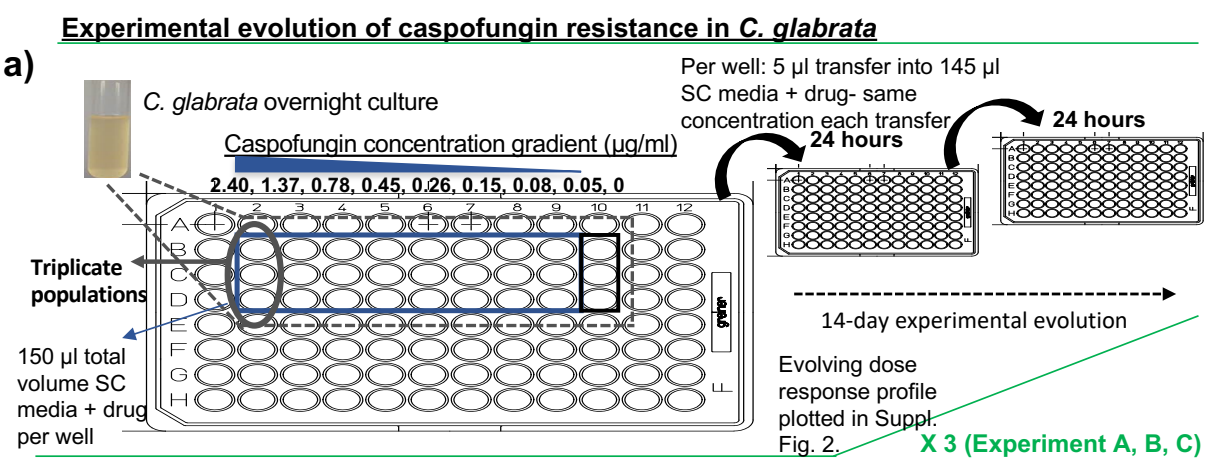
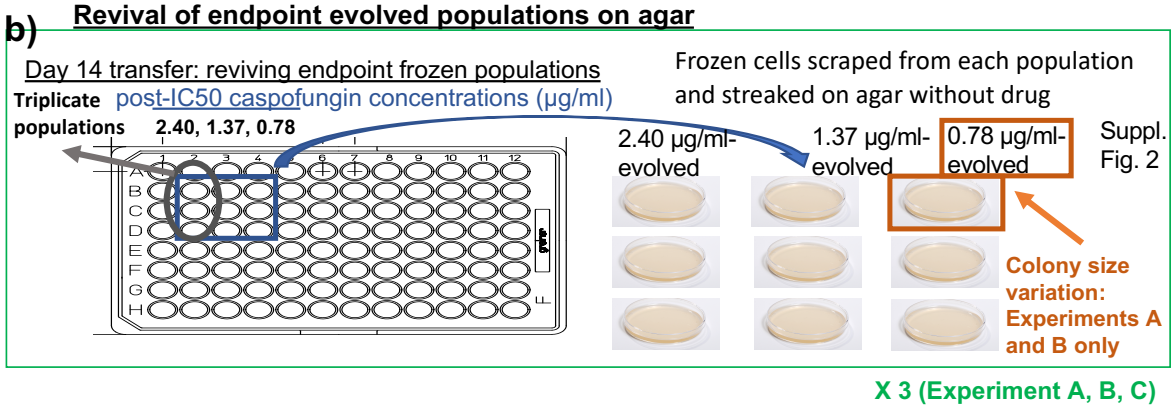
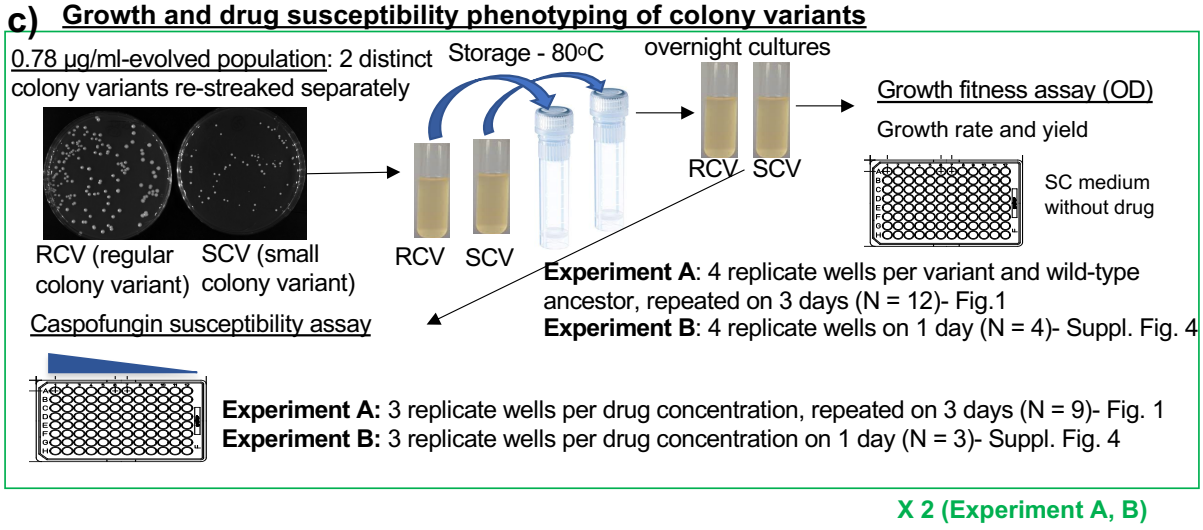
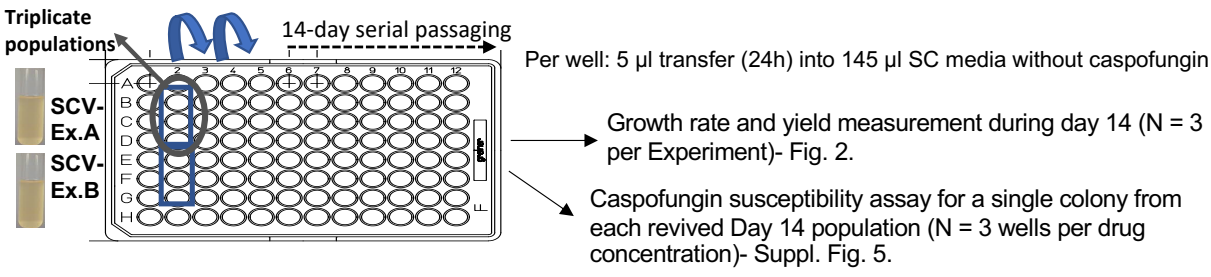
Supplementary Figures

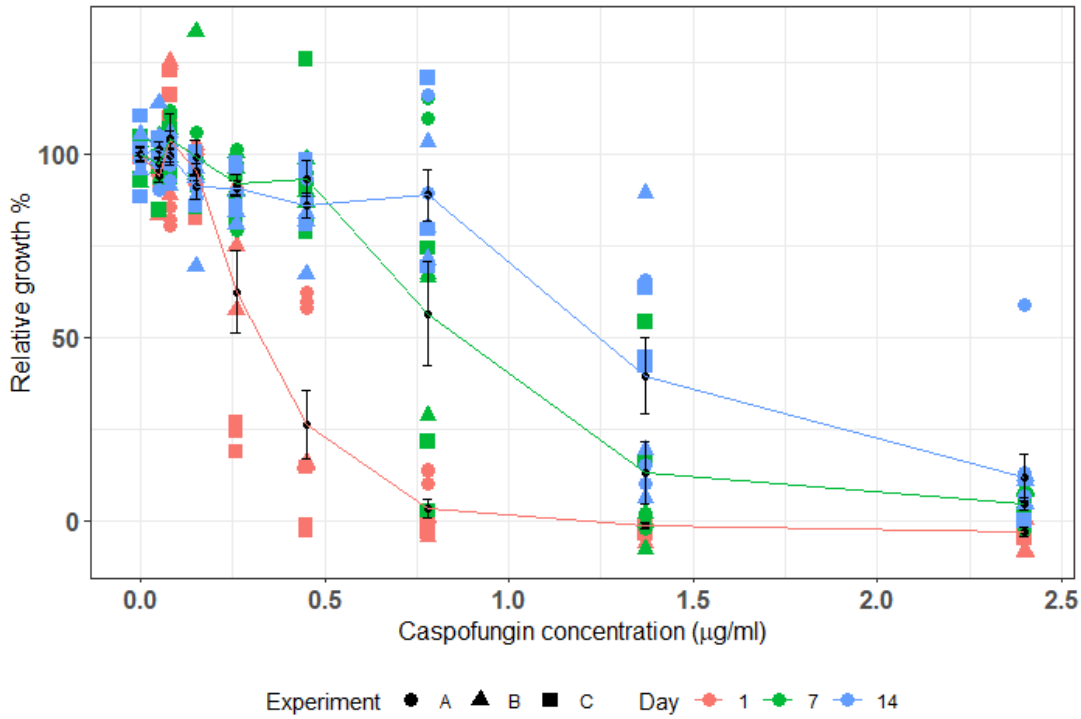




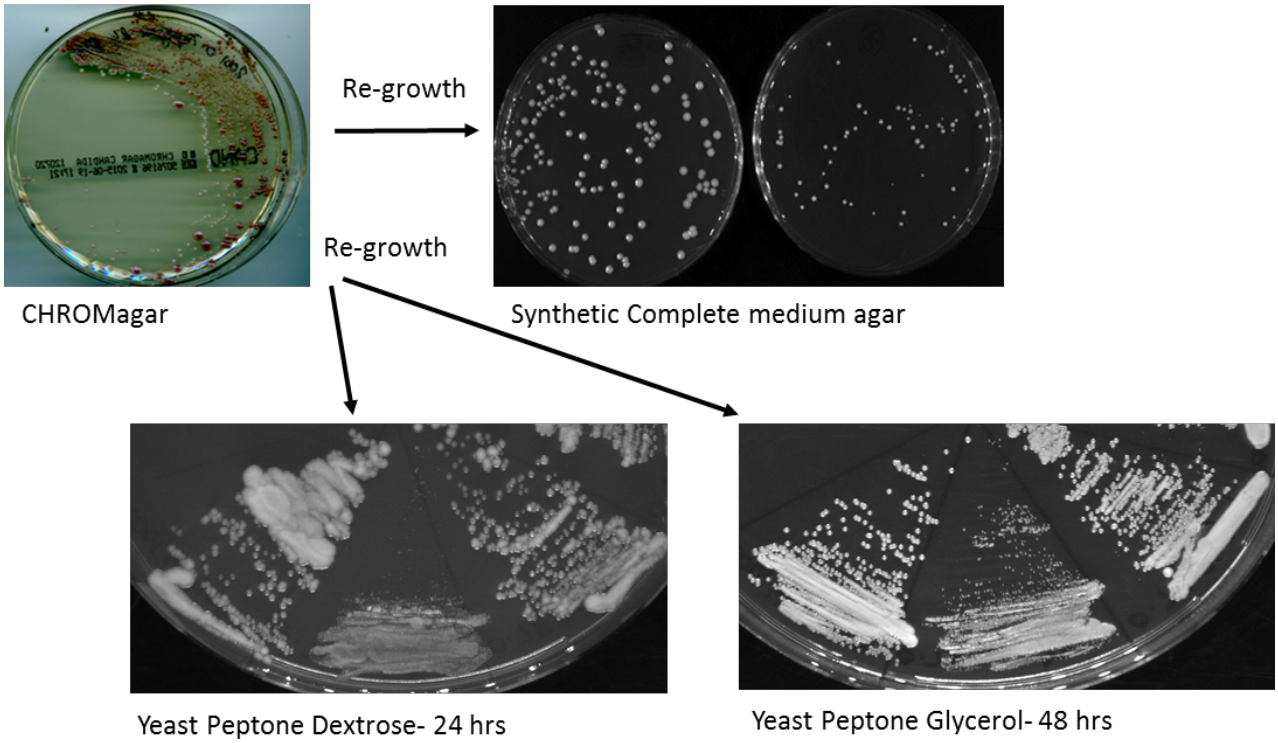




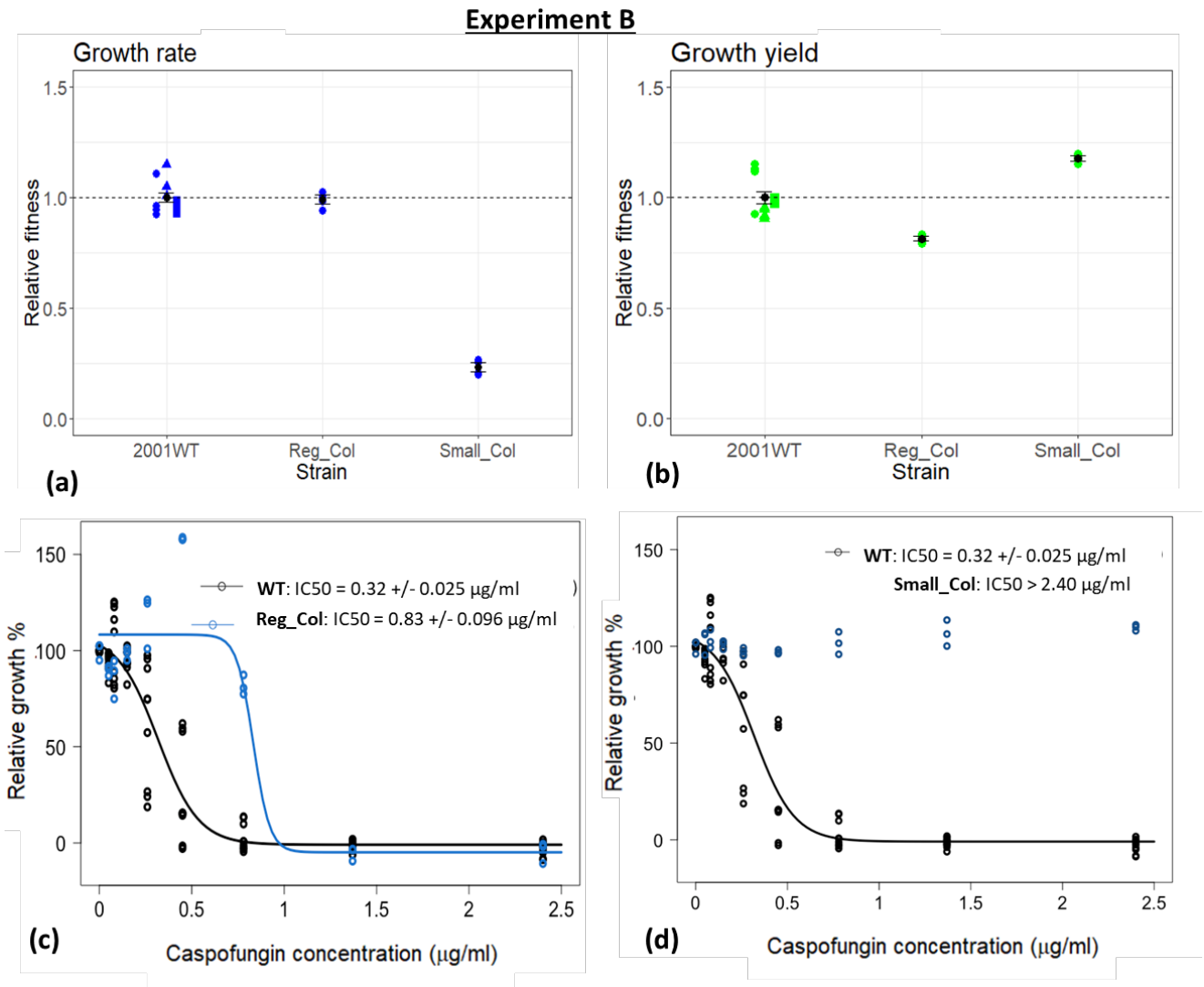
Supplementary Figure 1. Experimental design for evolution of caspofungin resistance in *C. glabrata* populations and phenotypic characterisation of diverse sub-population variants. (a) *C. glabrata* populations evolving on a gradient of caspofungin concentrations. Eight caspofungin concentrations representing a 1.75-fold dilution series were prepared, with a decreasing gradient from columns 2 to 9. Drug-free control wells were in column 10. Column 11 contained media controls. Triplicate populations (wells of plate) were evolved at each drug concentration, via serial passaging. At each transfer (24 hours), a new 96-well plate was prepared with identical layout. Following daily transfers, remaining volumes of all cell populations were mixed with glycerol and the plate was frozen at -80^{o}C. In total, 14 serial transfers were performed and the whole experiment was repeated three times (Experiments A, B, C). (b) Endpoint (day 14) populations were revived from frozen cultures that were evolved at the three highest (post-IC50) caspofungin concentrations. Populations were streaked on CHROMagar plates to control against bacterial contamination and qualitative differences in colony morphology were recorded. No colony size variation was detected in Experiment C. (c) A single colony of each size variant (RCV and SCV) was randomnly selected, that had been detected in a single population from each of Experiments A and B (evolved at 0.78 μg/ml). A freezer stock of each re-streaked colony variant was prepared, from which overnight cultures were prepared for future phenotypic analyses (growth fitness and caspofungin susceptibility assays). Separate overnight cultures of the colony variants were prepared for replicate measurements made on separate days. (d) To test for stability of the SCV phenotype isolated in each of Experiments A and B, triplicate populations were passaged in a 96-well plate, seeded from a single overnight culture prepared from the freezer stock of each previously isolated colony variant in (c). During the last transfer cycle (day 14), growth rate and yield measurements of all six populations were measured *in situ* via automated OD profiling. Freezer stocks of the six populations were prepared and caspofungin susceptibility assays were later performed by revival of the populations on agar and preparation of an overnight culture from a single randomnly selected colony per population.



Supplementary Figure 2. Changes in growth density of populations of *C. glabrata* strain 2001WT evolving on a gradient of caspofungin concentrations. 9 independent populations in total (3 populations in each of Experiments A, B and C) of *C. glabrata* were serially transferred daily in each of 8 caspofungin concentrations for 14 days. Relative growth % is the final optical density (24 h) of a drug-treated population, as a percentage of the average optical density of the no-drug treated populations. Data points are shown in different colours for days 1, 7 and 14. Different symbol shapes represent data from different Experiments (A, B and C). Mean relative growth across all 9 data points at each caspofungin concentration is plotted with standard error bars. Relative growth of drug-treated *C. glabrata* populations was significantly influenced by day ($p = 4.404e-12$) and caspofungin concentration ($p < 2.2e-16$).

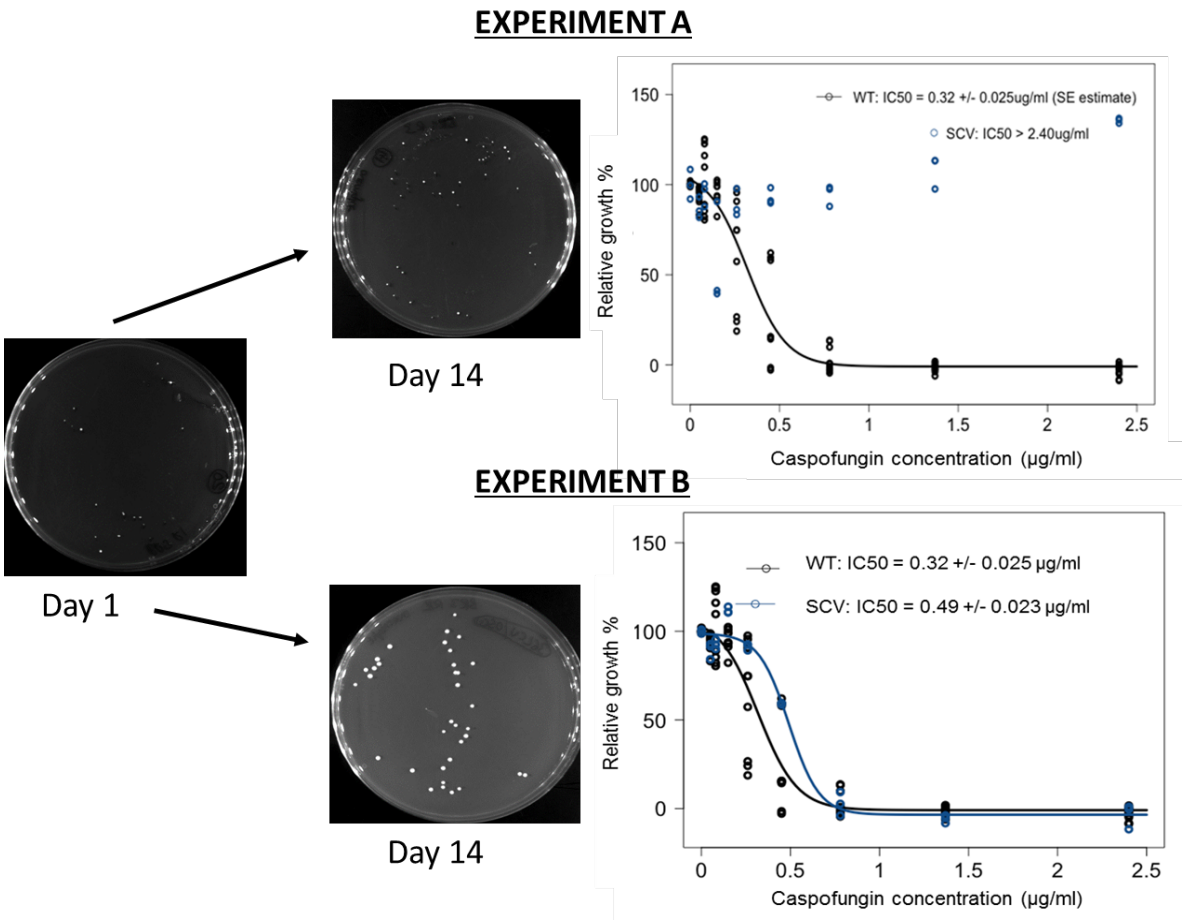


Supplementary Figure 3. Sub-population diversity in a drug-evolved population. Endpoint heterogeneity was shown in Experiment A for a single population after 14 days of transfers in 0.78 µg/ml caspofungin. The CHROMagar plate shows the revived population by re-streaking from frozen. All colonies (pink/purple) are *C. glabrata.* The two colony size variants (SCV) and (RCV) re-grew on Synthetic Complete medium agar when plated, following separate overnight culturing of each in liquid Synthetic Complete medium. Each colony variant could grow on Yeast Peptone Dextrose (YPD with 2% w/v glucose) and on Yeast Peptone Glycerol (YPG: same medium as YPD with 2% w/v glycerol instead of glucose) agar. Dextrose (glucose) can be respired and fermented whereas glycerol can only be respired. Colonies were streaked on plates in sectors: wild-type ancestor (2001WT); small colony (SCV); regular colony (RCV) (left to right sectors). Growth on YPD and YPG was also seen for the SCV and RCV variants isolated in Experiment B.

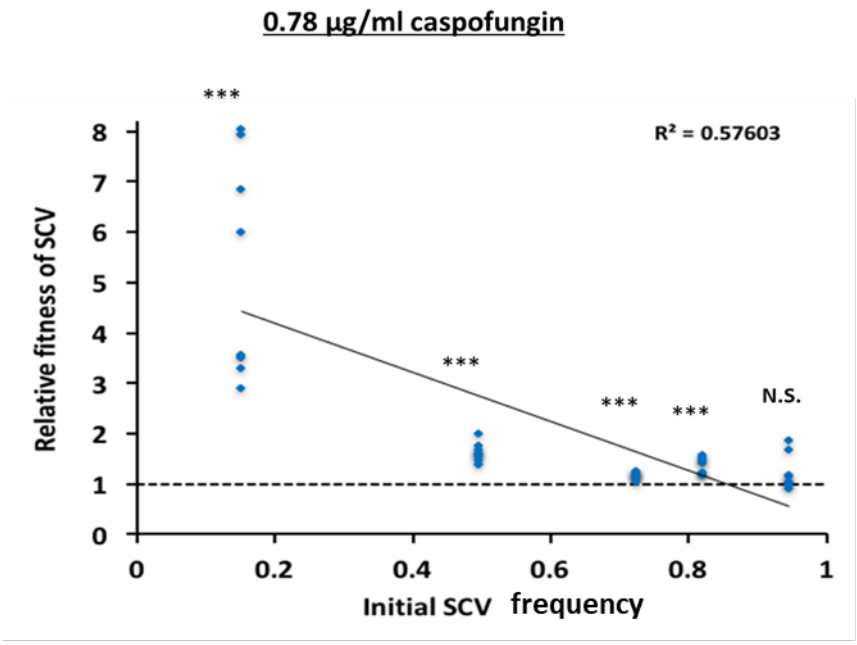


Supplementary Figure 4. Sub-population colony diversity in growth fitness and drug susceptibility in a single population evolved in 0.78 µg/ml caspofungin in Experiment B. Reg_Col (RCV) = regular-sized colony variant; Small_Col (SCV) = small colony variant; wild-type ancestral strain = 2001WT. Plots (a) (relative intrinsic growth rate) and (b) show growth in liquid medium over 24 hours in the absence of caspofungin. Growth values are calculated relative to average values of 2001WT, where a value of 1.0 shows no change relative to the ancestor. Black points and error bars overlaying each box represent mean and standard error. N = 12 for 2001WT (same data as in Figures 1 and 2) and N = 4 (well replicates on a single day) for the other two colony variants. Average relative growth rates (+/- SE): Reg_Col: 0.99 +/- 0.02; Small_Col: 0.23 +/- 0.02. Average relative growth yields (+/- SE): Reg_Col: 0.81 +/- 0.01; Small_Col: 1.18 +/- 0.01. Plots (c) and (d) show growth of populations on a gradient of caspofungin concentrations measured as final optical density after

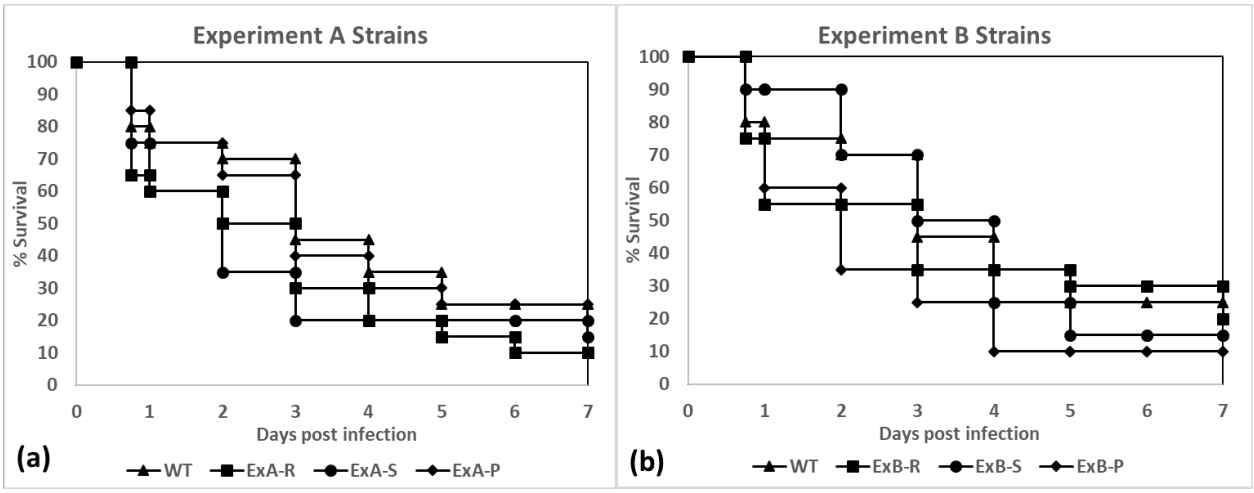
24-hour growth, as a percentage of average growth of the no-drug treated populations. N = 9 for the wild-type ancestor (same data as in Figure 1c and d) for each drug concentration. N = 3 (well replicates on a single day)) for the regular-sized and small colony variants for each drug concentration. Model-predicted (4-parameter logistic) IC50 values +/- SE of the estimated value are shown for each dose response.



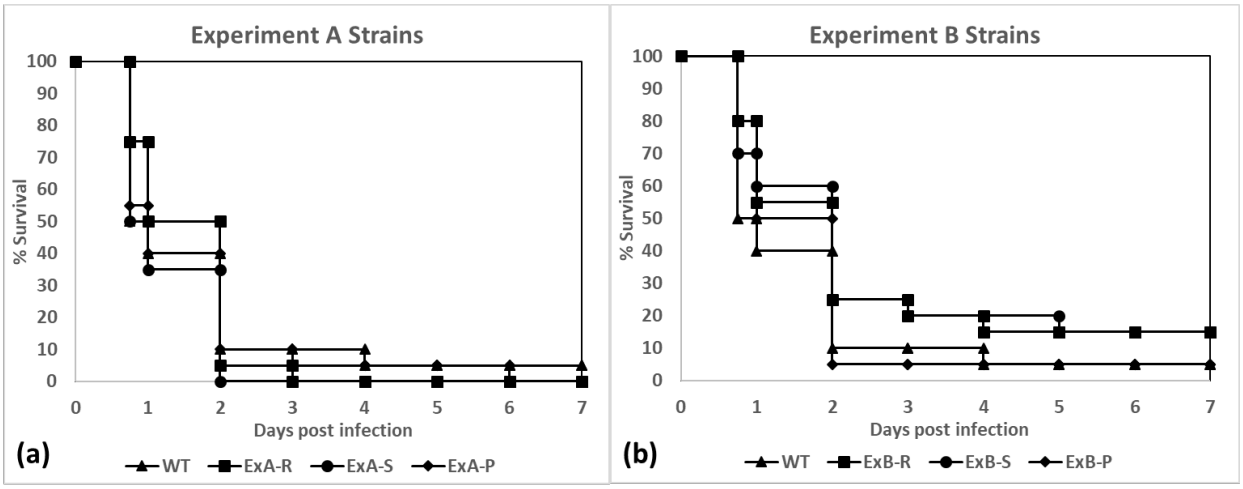
Supplementary Figure 5. Stability of the independently-isolated small colony phenotypes following serial passaging. The single isolated SCV from each of Experiments A and B was passaged in triplicate populations in the absence of caspofungin for 14 days. All three populations per colony variant showed highly similar endpoint (day 14) colony morphologies and population clone caspofungin susceptibilities. Data are presented for a single population and are representative of the triplicate populations per colony variant. The plate photographs show both SCVs starting with small colony morphology on day 1, which was maintained in passaged populations in Experiment A but reversion to wild-type colony size occurred across populations in Experiment B on day 14. Dose response profiles are presented for an endpoint (day 14) population clone from a single passaged replicate population of each colony variant. N = 3 (replicate culture wells of a microtiter plate) per drug concentration for each clone. N = 9 for each drug concentration of 2001WT, using the same data as in Figure 1c and d. The model-predicted IC50 values +/- SE of the estimated value are shown for each colony variant dose response.



Supplementary Figure 6. Competitive fitness of the SCV and RCV co-isolated from a single evolved population in Experiment A. Fitness of SCV (small colony variant) relative to the co-isolated RCV over 24-hour competition in the presence of caspofungin, calculated as the ratio of SCV/RCV Malthusian parameters [46]. N = 9 for each initial fraction. The black continuous line is the best-fit least-squares linear regression, with the R-squared correlation coefficient shown. The dotted line indicates a relative fitness of 1. Asterisks indicate p values of significance from one-sample two-tailed t-tests. N.S. = non-significant ($p > 0.05$). (a) T-test results for initial fractions: frac 0.15: $t(7) = 5.5315$, $p = 0.0008768$; frac 0.49: $t(8) = 10.796$, $p = 4.777e\text{-}06$; frac 0.72: $t(8) = 8.0344$, $p = 4.234e\text{-}05$; frac 0.82: $t(8) = 6.9143$, $p = 0.0001227$; frac 0.95: $t(8) = 1.9116$, $p = 0.09231$.

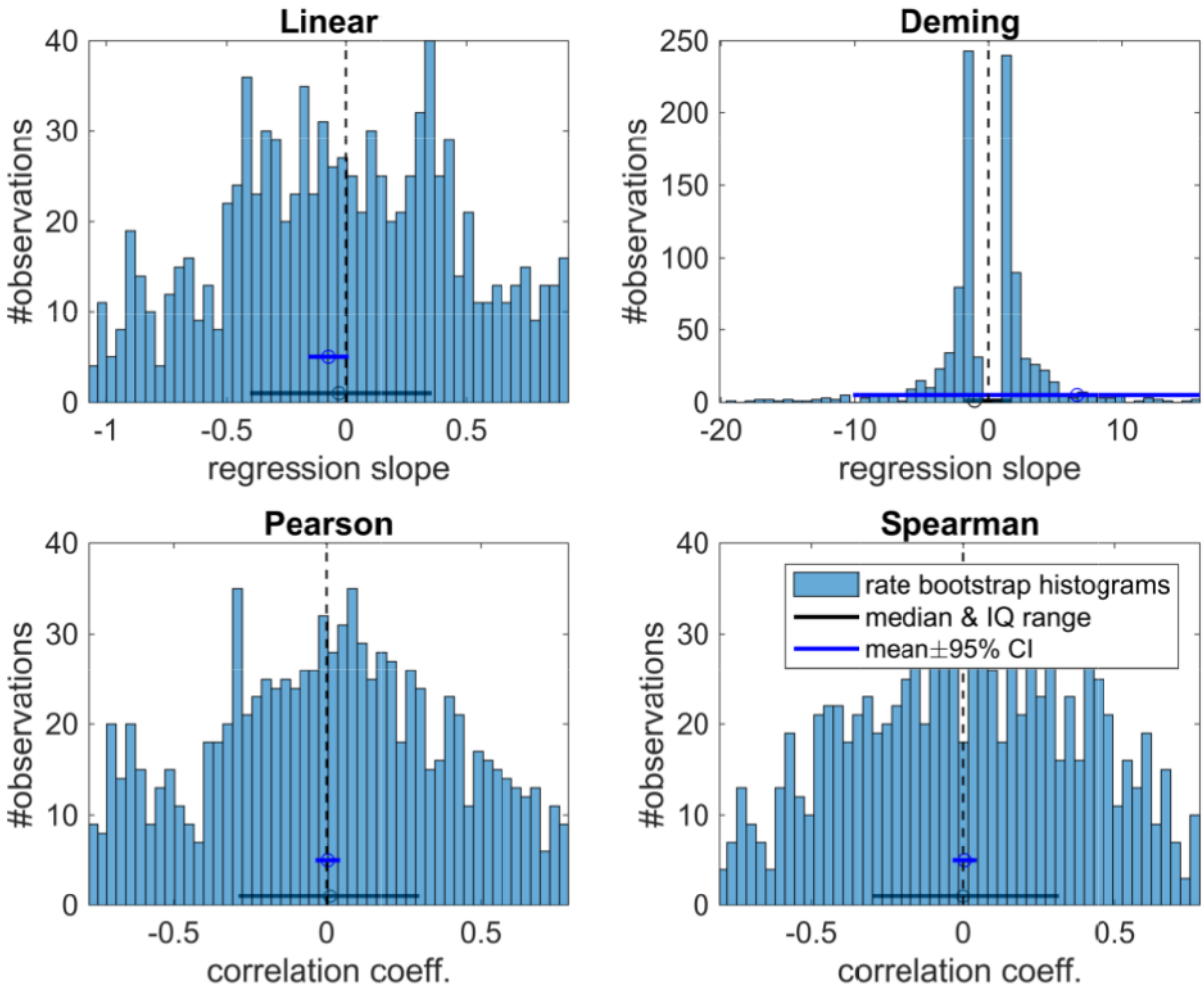


Supplementary Figure 7. Virulence of *C. glabrata* wild-type ancestral, small and regular colony size variants in *G. mellonella* larvae (second replicate study). Survival of groups of 20 *G. mellonella* wax moth larvae injected with 2.5 x 10^6 CFU/larva per strain over 7-day incubation at 37°C. (a) WT = 2001WT ancestral strain, ExA-R = Experiment A regular colony variant, ExA-S = Experiment A small colony variant, ExA-P = Experiment A passaged small colony variant. The small colony variant was virulent in *G. mellonella* both before and after passaging without caspofungin. Mean larval survival times were 2.69 ± 0.51 days (ExA-S) and 3.56 ± 0.51 days (ExA-P) respectively, and we found no significant differences from 2001WT (3.70 ± 0.51 days; log-rank test p-values = 0.163 (ExA-S); 0.5871 (ExA-P)) or the co-isolated regular colony variant (2.76 ± 0.47 days; log-rank test p-values = 0.9802 (ExA-S); 0.3228 (ExA-P)). (b) ExB-S = Experiment B small colony variant, ExB-R = Experiment B regular colony variant, ExB-P = Experiment B passaged small colony variant (revertant). We found no significant difference in *G. mellonella* mean survival times of the “revertant” small colony variant when comparing states before (3.63 ± 0.41 days) and after (2.44 ± 0.42 days) loss of the phenotype (log-rank test, p = 0.0786). No significant differences in mean larval survival time occurred between 2001WT and either ExB-S (p = 0.7177) or ExB-P (p = 0.0785), nor between ExB-R (3.34 ± 0.59 days) and either ExB-S (p = 0.8439) or ExB-P (p = 0.3078).



Supplementary Figure 8. Virulence of *C. glabrata* wild-type ancestral, small and regular colony size variants in *G. mellonella* larvae (third replicate study). Survival of groups of 20 *G. mellonella* wax moth larvae injected with 2.5 x 10^6 CFU/larva per strain over 7-day incubation at 37°C. (a) WT = 2001WT ancestral strain, ExA-R = Experiment A regular colony variant, ExA-S = Experiment A small colony variant, ExA-P = Experiment A passaged small colony variant. The small colony variant was virulent in *G. mellonella* both before and after passaging without caspofungin. Mean larval survival times (+/- SE) were 1.23 ± 0.13 days and 1.54 ± 0.27 days respectively, and we found no significant differences from 2001WT (1.63 ± 0.33 days; log-rank test p-values = 0.4743 (ExA-S); 0.808 (ExA-P)) or the co-isolated regular colony variant (1.49 ± 0.15 days; log-rank test p-values = 0.1755 (ExA-S); 0.7745 (ExA-P)). (b) ExB-S = Experiment B small colony variant, ExB-R = Experiment B regular colony variant, ExB-P = Experiment B passaged small colony variant (revertant). We found no significant difference in *G. mellonella* mean survival times of the “revertant” small colony variant when comparing states before (2.48 ± 0.48 days) and after (1.70 ± 0.30 days) loss of the phenotype (log-rank test, p = 0.3425). No significant differences in mean larval survival time occurred between 2001WT and either ExB-S (p = 0.14) or ExB-P (p = 0.4572), nor between ExB-R (2.40 ± 0.47 days) and either ExB-S (p = 0.9597) or ExB-P (p = 0.3698).

(a)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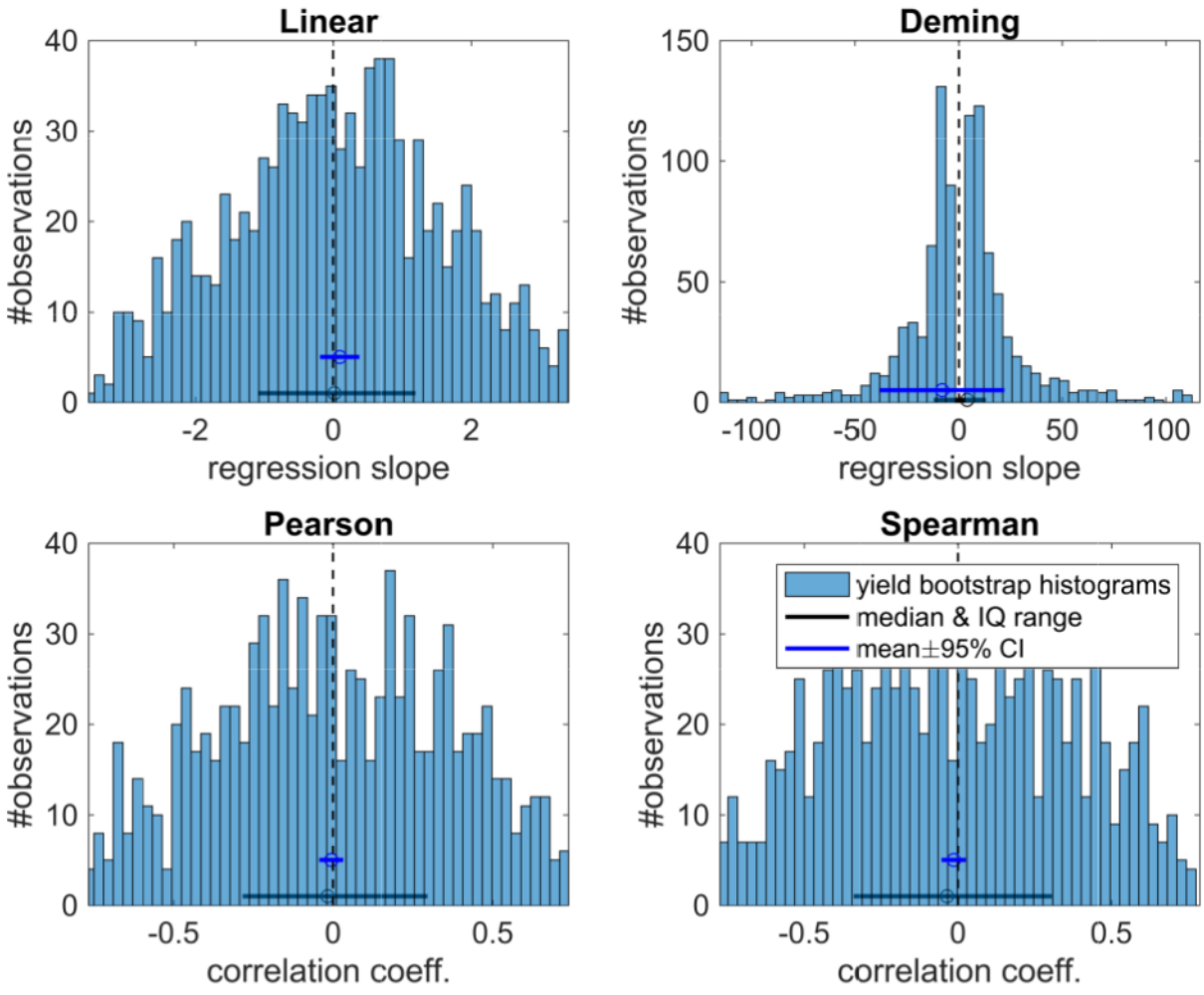
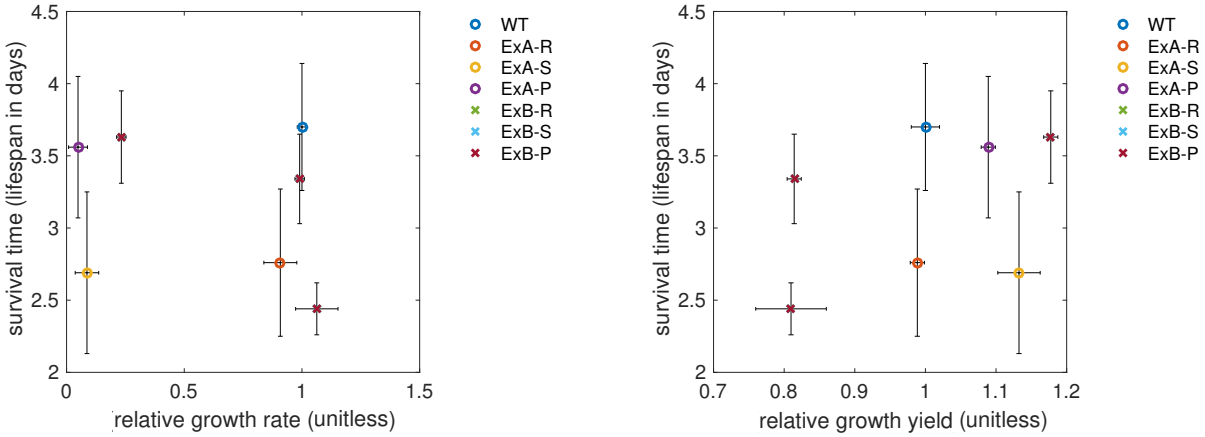


Supplementary Figure 9. Growth rate, growth yield and virulence (first replicate study). Analysis of data presented in Figure 4 where growth traits were plotted against larval survival time for all strains from Experiments A and B, including regular colony variants and small colony variants before and after passaging. Bootstrapping was performed for both Linear and Deming regressions, in addition to both Pearson and Spearman correlations. None of these detected a correlation between relative growth rates and larval survival times (measure of virulence) (a) nor between relative growth yield and virulence (b).

(a)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relative growth rate (unitless)
relative growth yield (unitless)
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ExA-S
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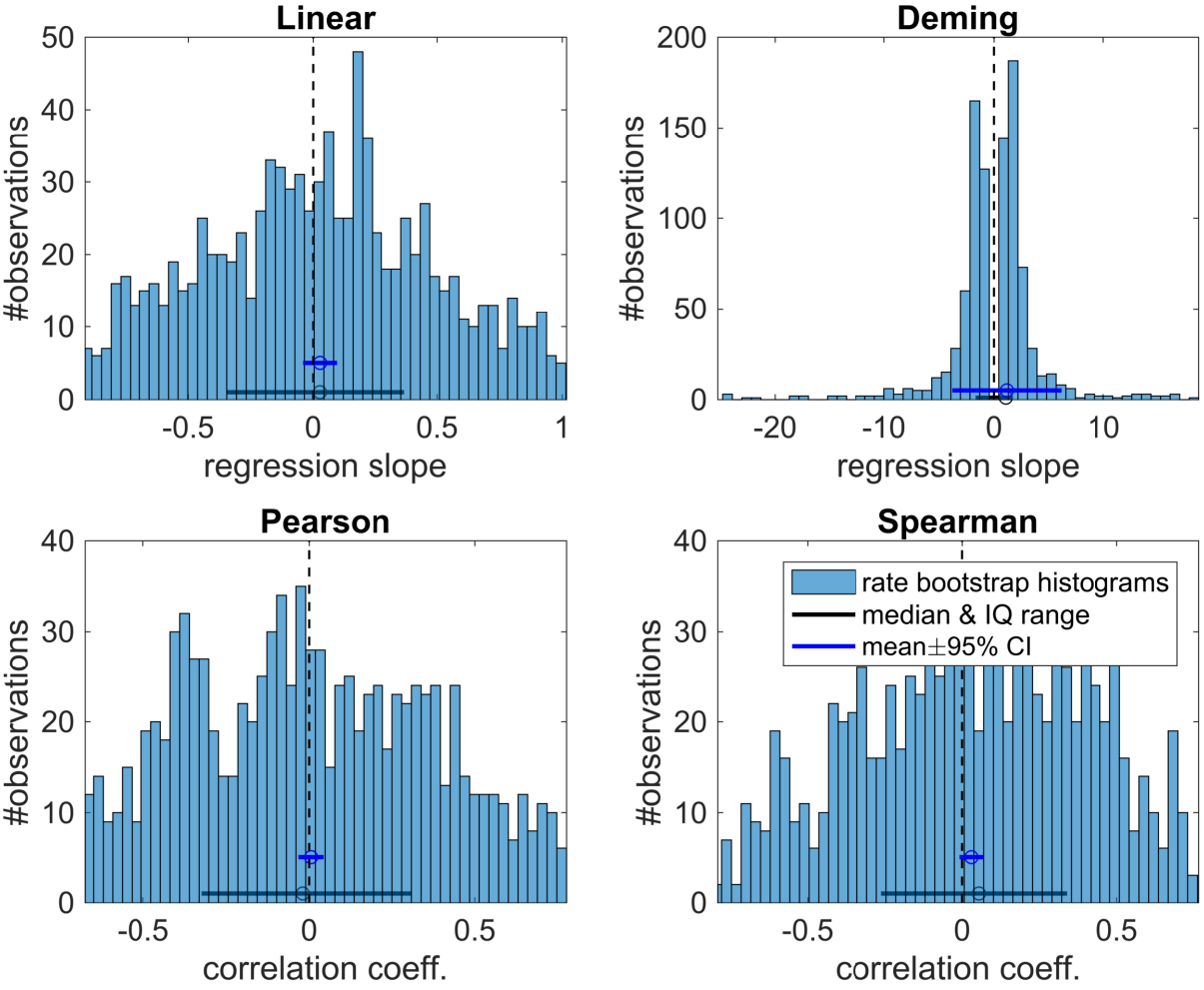
(b)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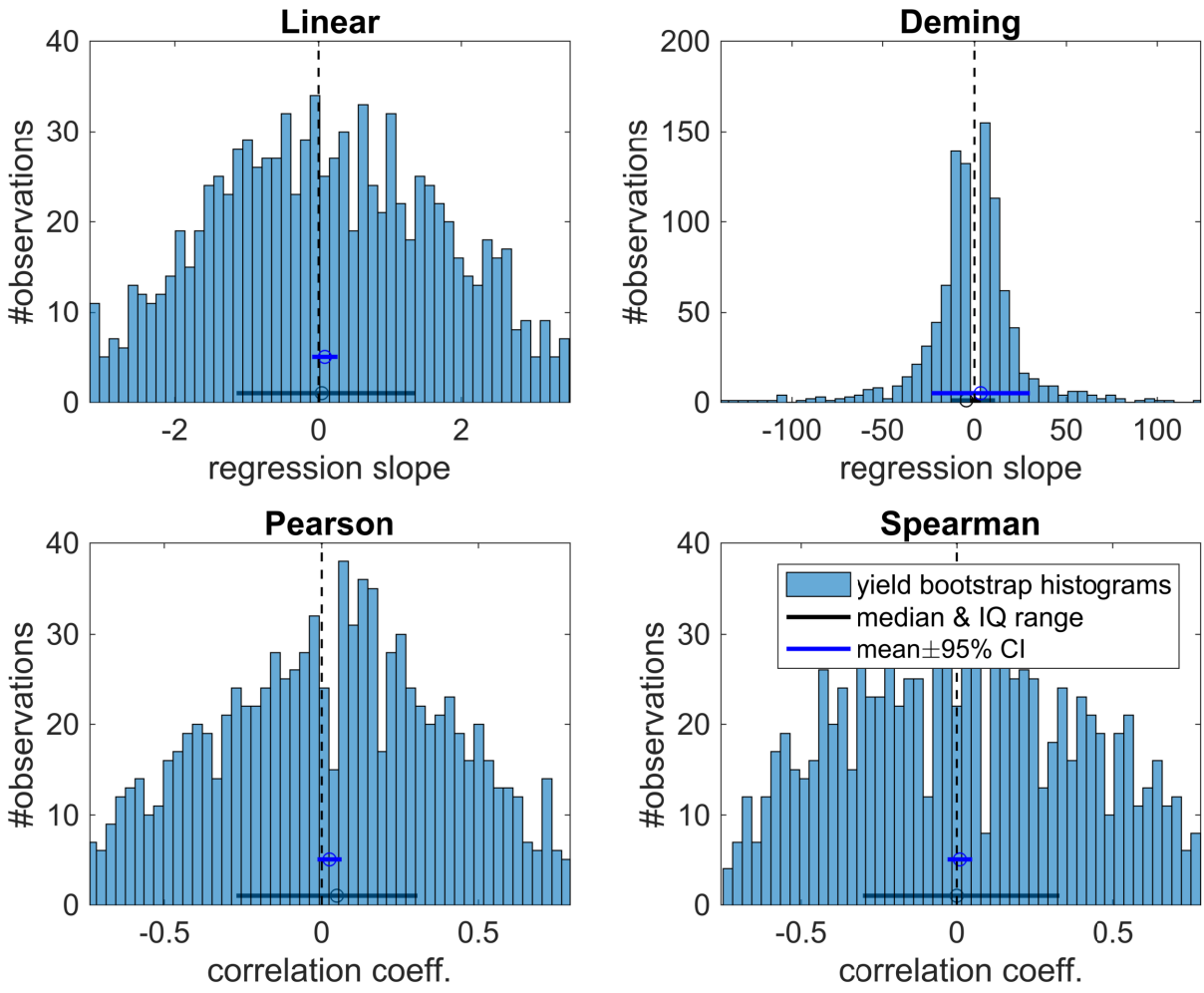
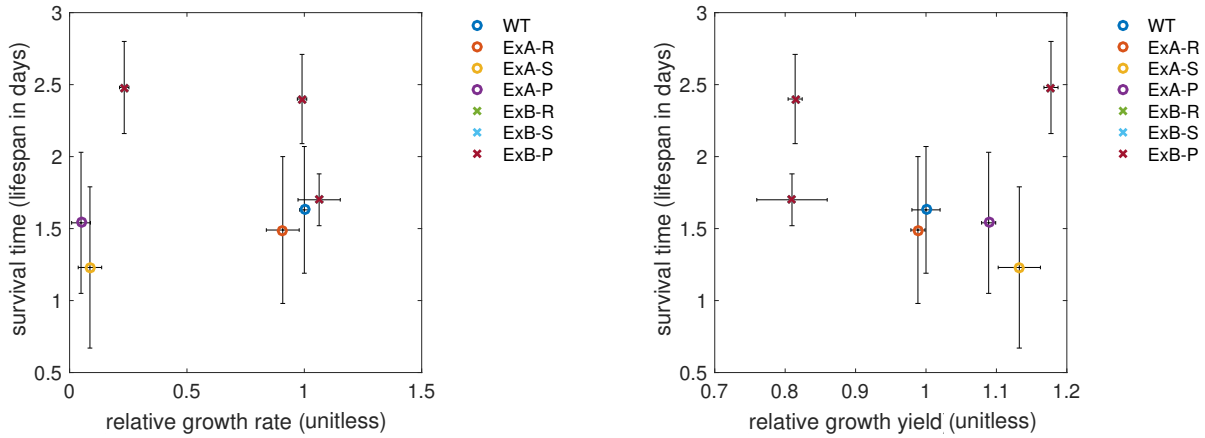


Supplementary Figure 10. Growth rate, growth yield and virulence (second replicate study). (a) Growth traits are plotted for all strains from Experiments A and B, including regular colony variants and small colony variants before and after passaging. Data is combined from Figures 1a, b, 2 and Supplementary Figure 7. Plotted points represent mean values +/- SE. Growth rate and yield are plotted relative to the wild-type ancestral strain (2001WT). Strains are labelled as - Experiment A strains: ExA-R (regular colony variant); ExA-S (small colony variant); ExA-P (passaged “stable” small colony). Experiment B strains: ExB-R (regular colony variant); ExB-S (small colony variant); ExB-P (passaged “unstable” small colony). Bootstrapping was performed for both linear and Deming regressions, in addition to both Pearson and Spearman correlations. None of these detected a correlation between relative growth rates and larval survival times (measure of virulence) (b) nor between relative growth yield and virulence (c).

(a)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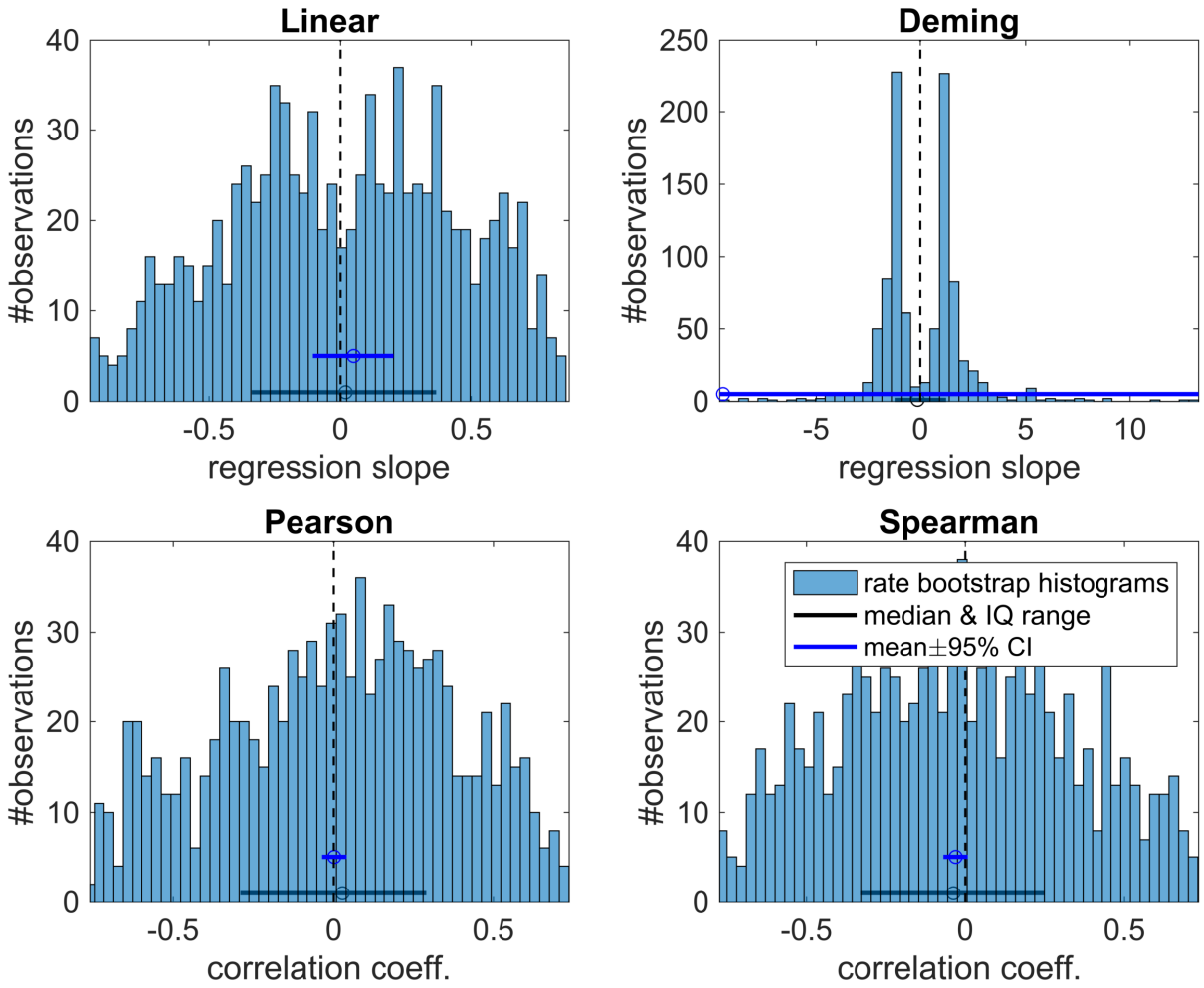
(b)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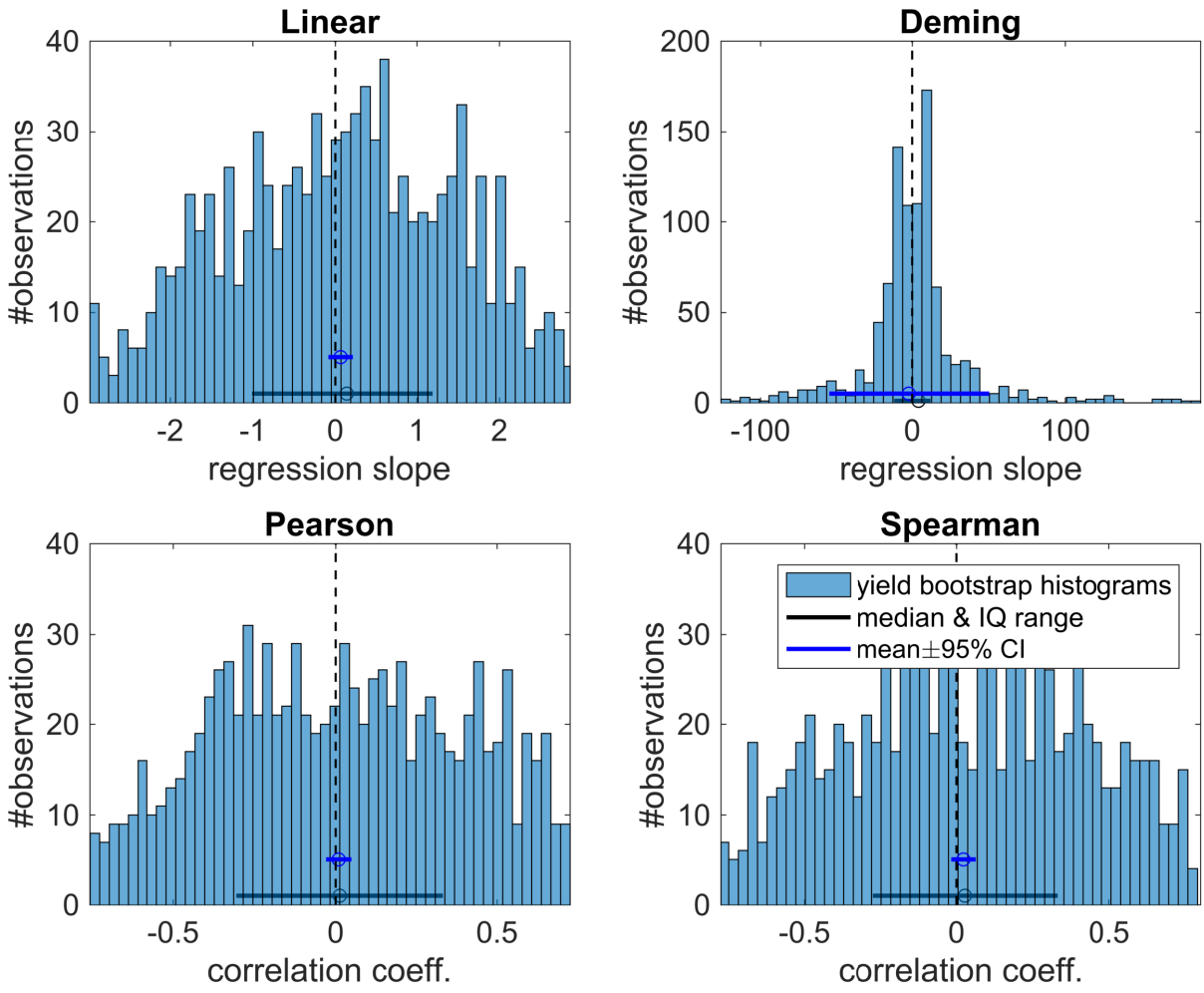

Supplementary Figure 11. Growth rate, growth yield and virulence (third replicate study). (a) Growth traits are plotted for all strains from Experiments A and B, including regular colony variants and small colony variants before and after passaging. Data is combined from Figures 1a, b, 2 and Supplementary Figure 8. Plotted points represent mean values +/- SE. Growth rate and yield are plotted relative to the wild-type ancestral strain (2001WT). Strains are labelled as - Experiment A strains: ExA-R (regular colony variant); ExA-S (small colony variant); ExA-P (passaged “stable” small colony). Experiment B strains: ExB-R (regular colony variant); ExB-S (small colony variant); ExB-P (passaged “unstable” small colony). Bootstrapping was performed for both linear and Deming regressions, in addition to both Pearson and Spearman correlations. None of these detected a correlation between relative growth rates and larval survival times (measure of virulence) (b) nor between relative growth yield and virulence (c).