Electronic Supplementary Material

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

Sarah J.N. Duxbury[1,2], Steven Bates[1], Robert E. Beardmore[1], Ivana Gudelj[1]

[1]Department of Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, Devon EX4 4QD, UK; [2]Laboratory of Genetics, Wageningen University, 6708 PB Wageningen, The Netherlands.

Corresponding author: i.gudelj@exeter.ac.uk

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.

References

1. Kitada K, Yamaguchi E, Arisawa M, 1995. Cloning of the *Candida glabrata TRP1* and *HIS3* genes, and construction of their disruptant strains by sequential integrative transformation. *Gene* 165, 203-206. (doi:10.1016/0378-1119(95)00552-H)

2. Espinel-Ingroff A, Arendrup MC, Pfaller MA, Bonfietti LX, Bustamante B, Canton E, Chryssanthou E, Cuenca-Estrella M, Dannaoui E, Fothergill A, *et al*. 2013 Interlaboratory variability of caspofungin MICs for *Candida* spp. Using CLSI and EUCAST methods: Should the clinical laboratory be testing this agent? *Antimicrob. Agents. Chemother.* **57**, 5836-5842. (doi:10.1128/AAC.01519-13)

3. Bates D, Maechler M, Bolker B, Walker S. 2015 Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1-48. (doi:10.18637/jss.v067.i01)

4. R Development Core Team. 2017 *R: a language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing. See https://www.R-project.org/.

5. MacLean RC, Hall AR, Perron GG, Buckling A. 2010 The population genetics of antibiotic resistance: Integrating molecular mechanisms and treatment contexts. *Nat. Rev. Genet.* **11**, 405-414. (doi:10.1038/nrg2778)

6. Thompson GR, Wiederhold NP, Vallor AC, Villareal NC, Lewis JS, Patterson TF. 2008 Development of caspofungin resistance following prolonged therapy

for invasive candidiasis secondary to *Candida glabrata* infection. *Antimicrob. Agents Chemother.* **52**, 3783-3785. (doi: 10.1128/AAC.00473-08)

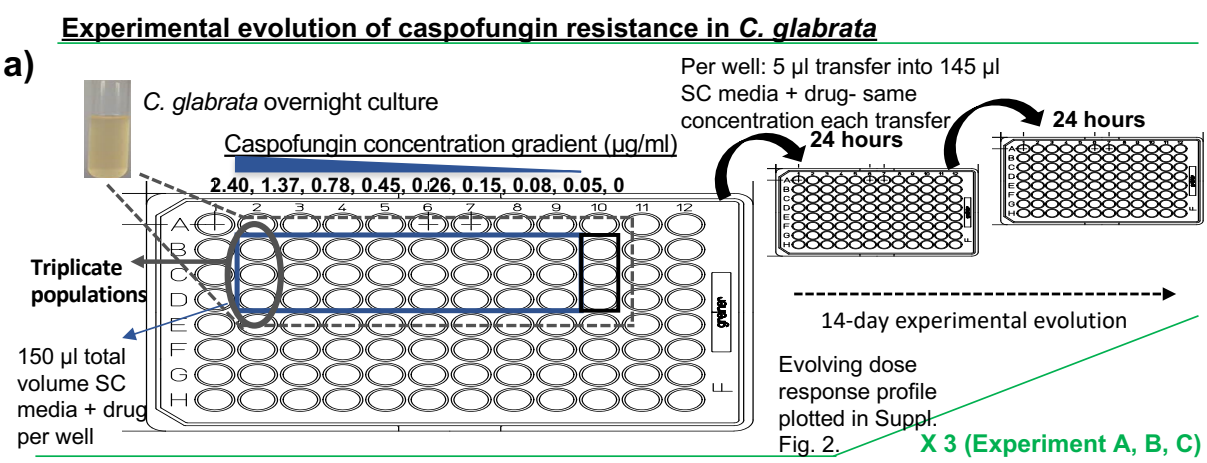
7. Zimbeck AJ, Iqbal N, Ahlquist AM, Farley MM, Harrison LH, Chiller T, Lockhart SR. 2010 *FKS* mutations and elevated echinocandin MIC values among *Candida glabrata* isolates from U.S. population-based surveillance. *Antimicrob. Agents Chemother.* **54**, 5042-5047. (doi: 10.1128/AAC.00836-10)

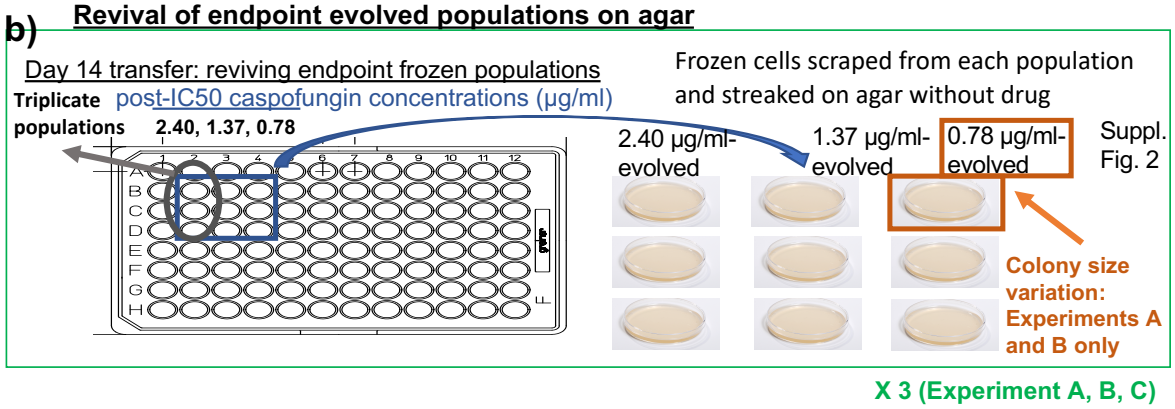
8. Singh-Babak SD, Babak T, Diezmann S, Hill JA, Xie JL, Chen YL, Poutanen SM, Rennie RP, Heitman J, Cowen LE. 2012 Global analysis of the evolution and mechanism of echinocandin resistance in *Candida glabrata*. *PLoS Pathog.* **8**, e1002718. (doi:10.1371/journal.ppat.1002718)

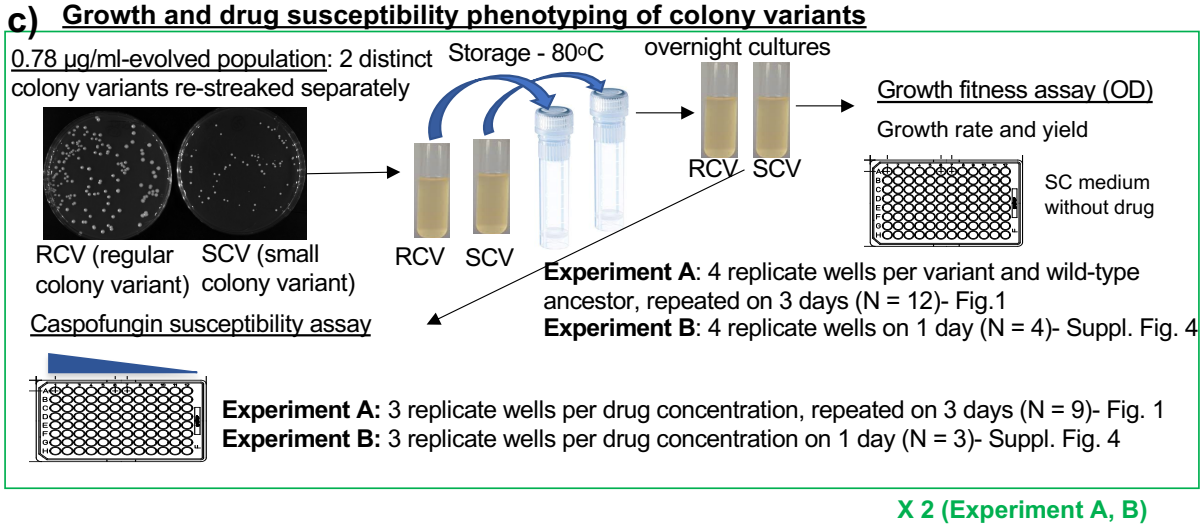
9. Kumar S, Stecher G, Tamura K. 2016 MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol*. **33**, 1870-1874. (doi: 10.1093/molbev/msw054)

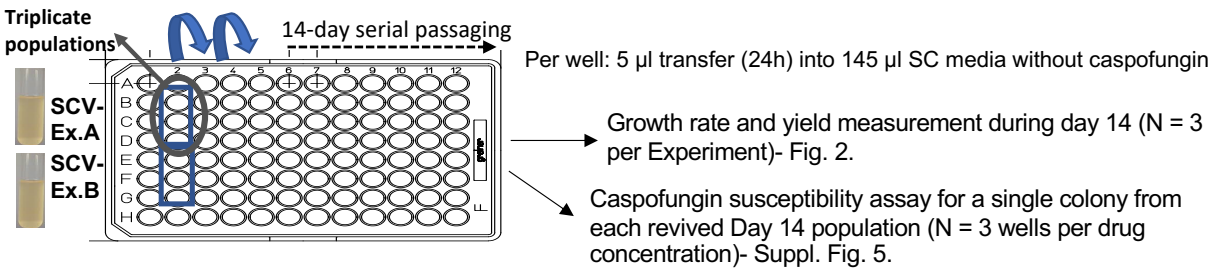
10. Zaiontz C. 2015 *Real Statistics Using Excel.* www.real-statistics.com

Supplementary Figures

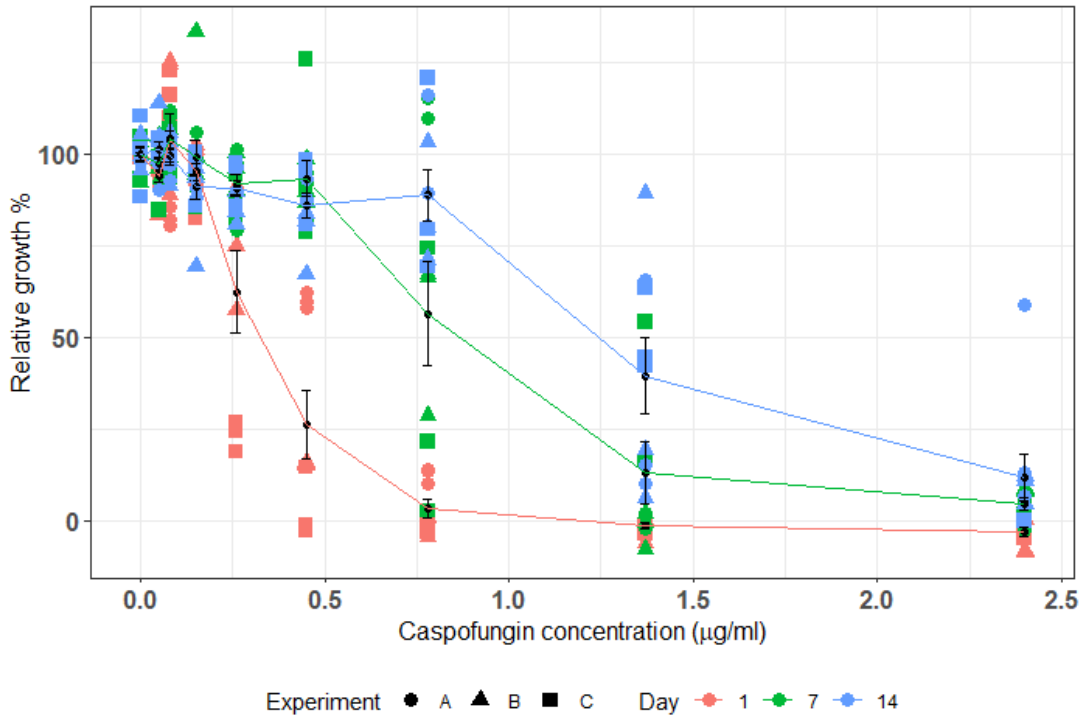




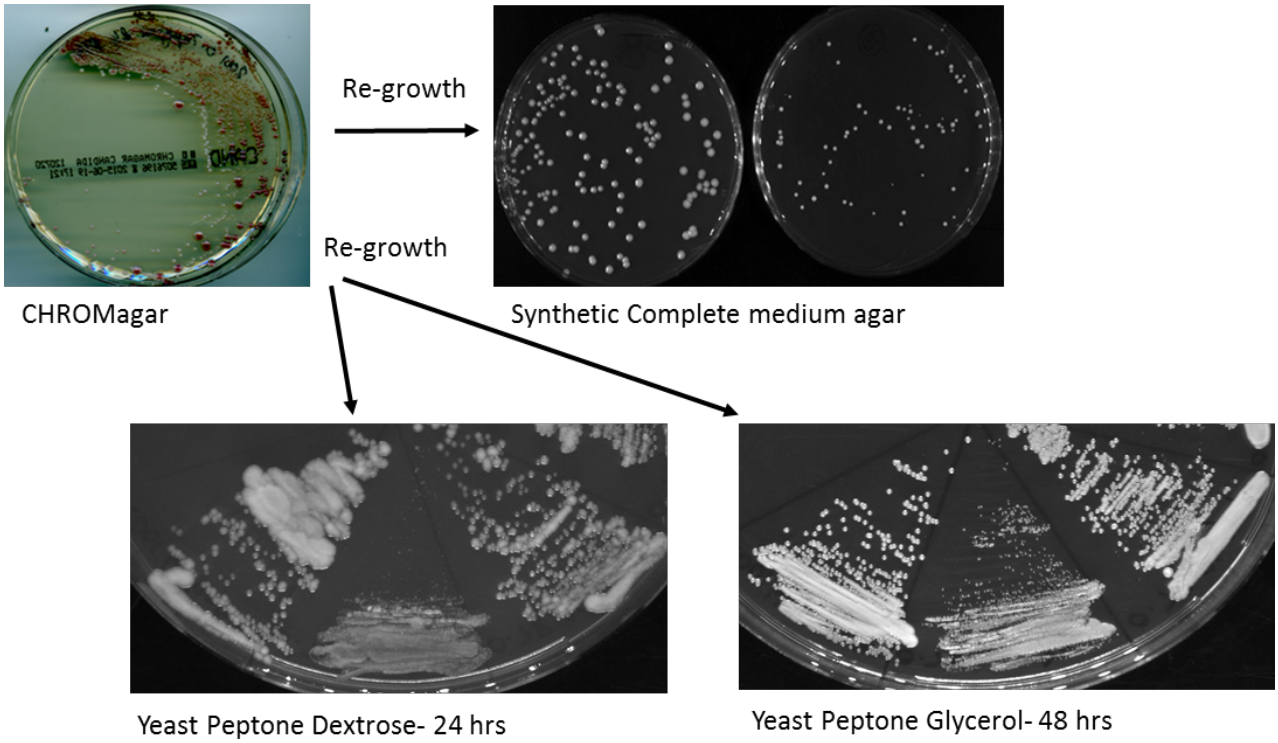




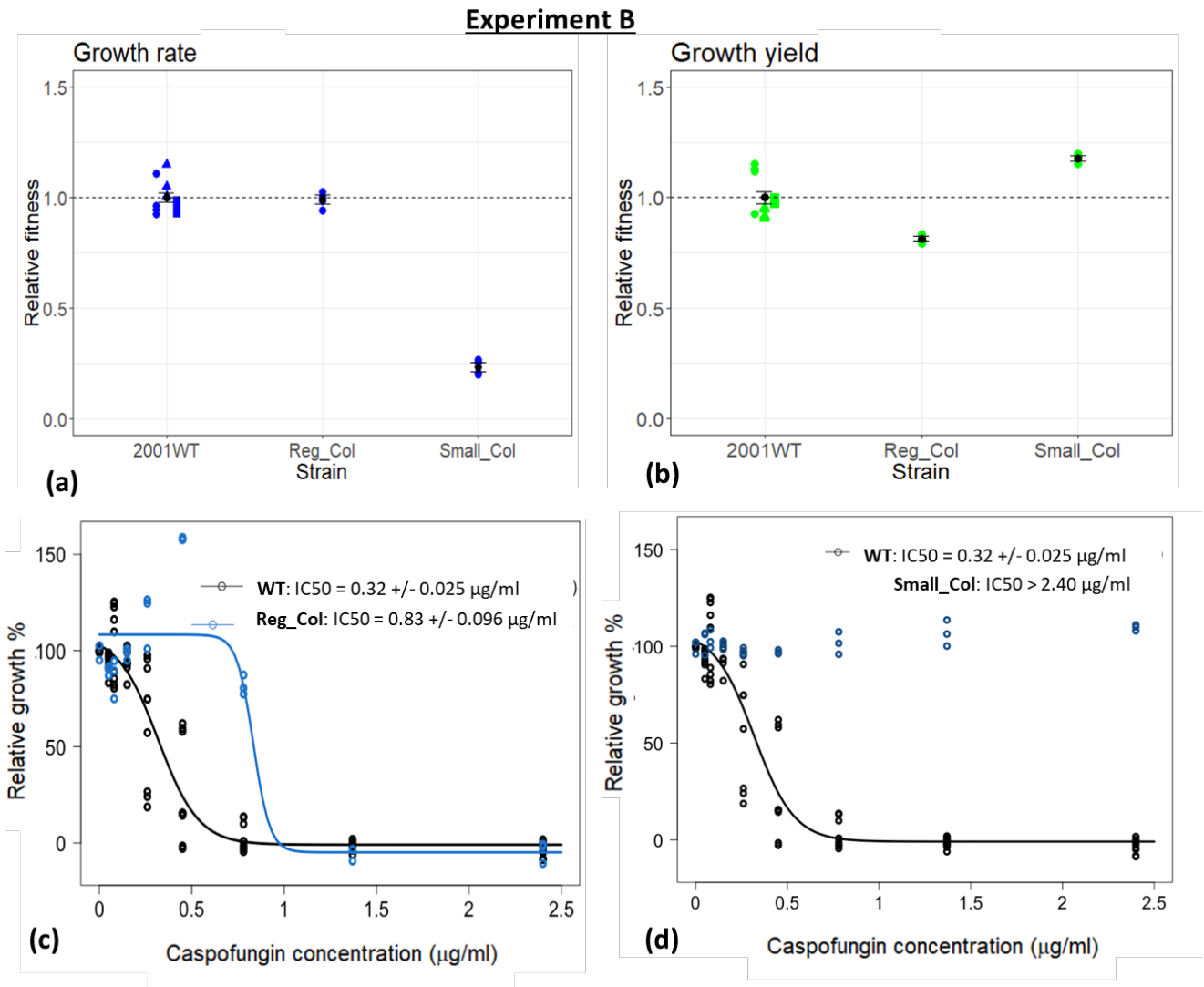
Supplementary Figure 1. Experimental design for evolution of caspofungin resistance in *C. glabrata* populations and phenotypic characterisation of diverse sub-population variants. (a) *C. glabrata* populations evolving on a gradient of caspofungin concentrations. Eight caspofungin concentrations representing 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 ($p < 2.2e-16$).

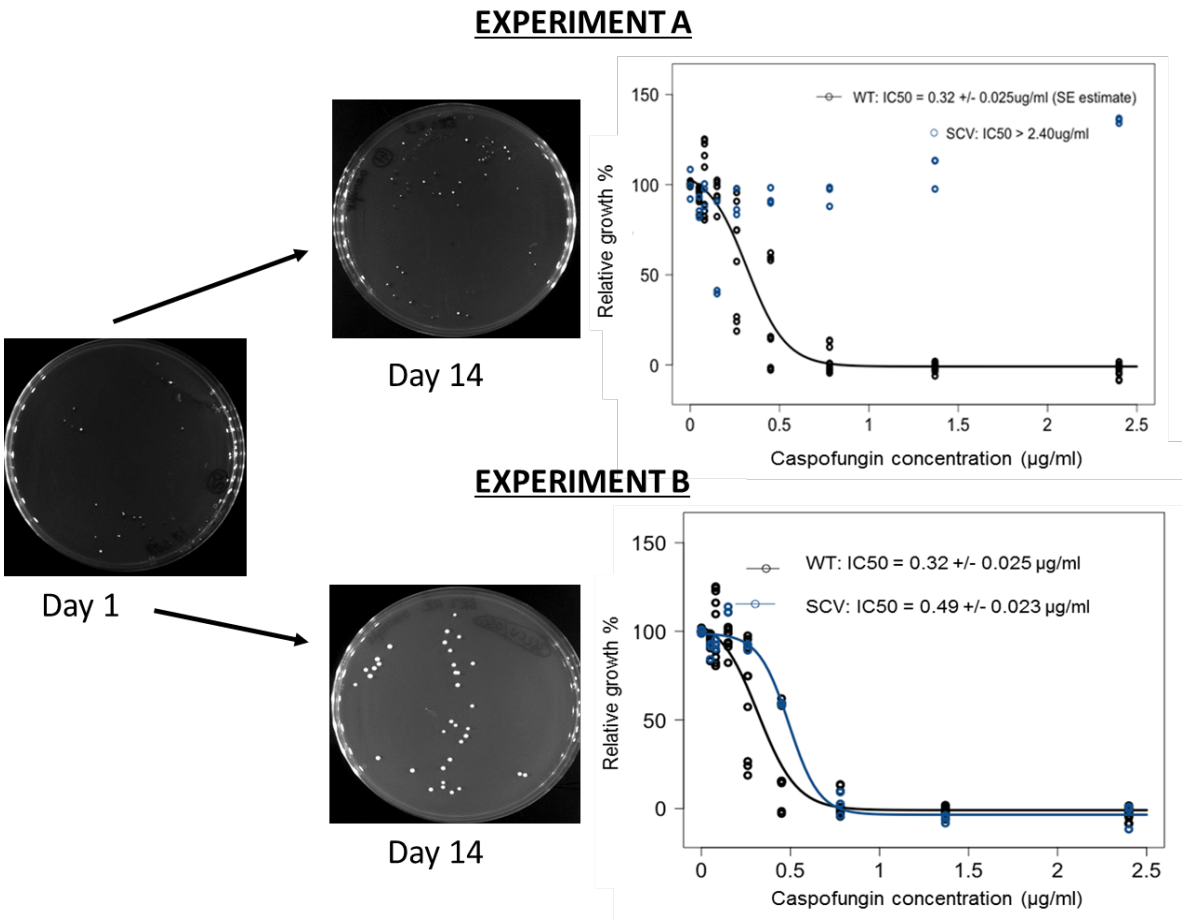


Supplementary Figure 3. Sub-population diversity in a drug-evolved population. Endpoint heterogeneity was shown in Experiment A for a single population after 14 days of transfers in 0.78 µg/ml caspofungin. The CHROMagar plate shows the revived population by re-streaking from frozen. All colonies (pink/purple) 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.

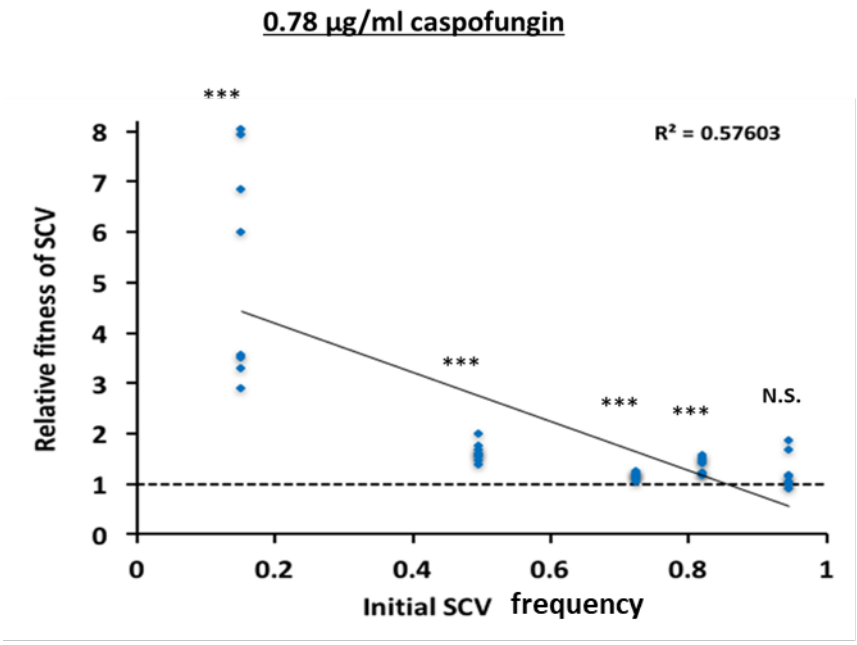


Supplementary Figure 4. Sub-population colony diversity in growth fitness and drug susceptibility in a single population evolved in 0.78 µg/ml caspofungin in Experiment B. Reg_Col (RCV) = regular-sized colony variant; Small_Col (SCV) = small colony variant; wild-type ancestral strain = 2001WT. Plots (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

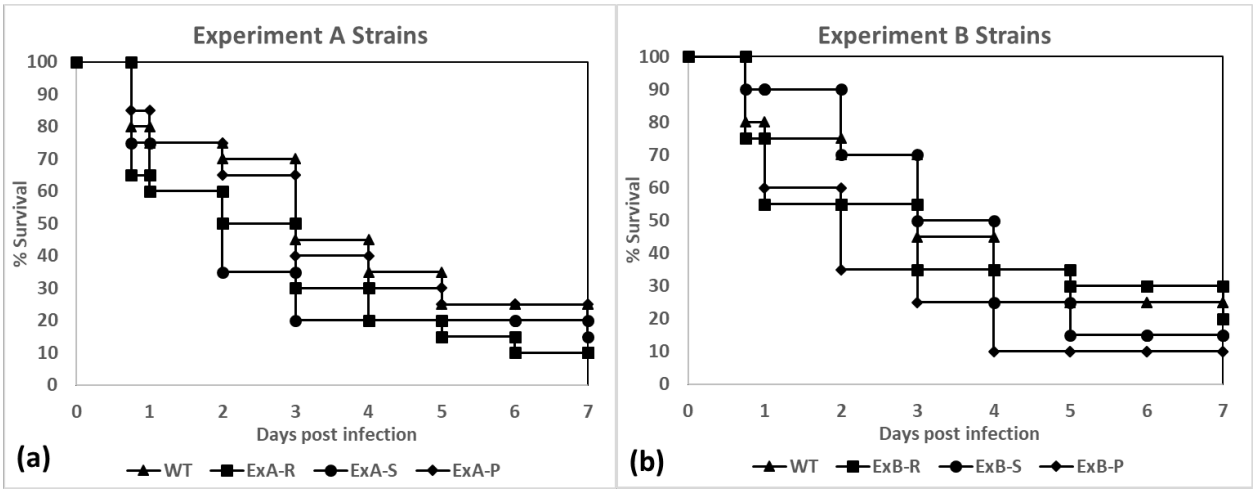
24-hour growth, as a percentage of average growth of the no-drug treated populations. N = 9 for the wild-type ancestor (same data as in Figure 1c and d) for each drug concentration. N = 3 (well replicates on a single day)) for the regular-sized and small colony variants for each drug concentration. Model-predicted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



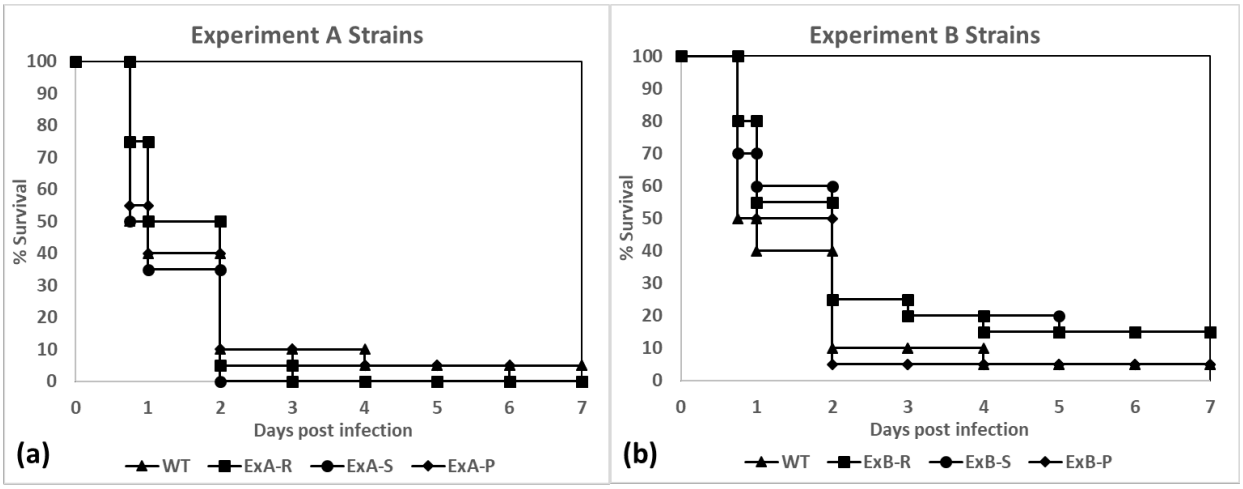
Supplementary Figure 5. Stability of the independently-isolated small colony phenotypes following serial passaging. The single isolated SCV from each of Experiments A and B was passaged in triplicate populations in the absence of caspofungin for 14 days. All three populations per colony variant showed 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: $t(8) = 1.9116$, $p = 0.09231$.

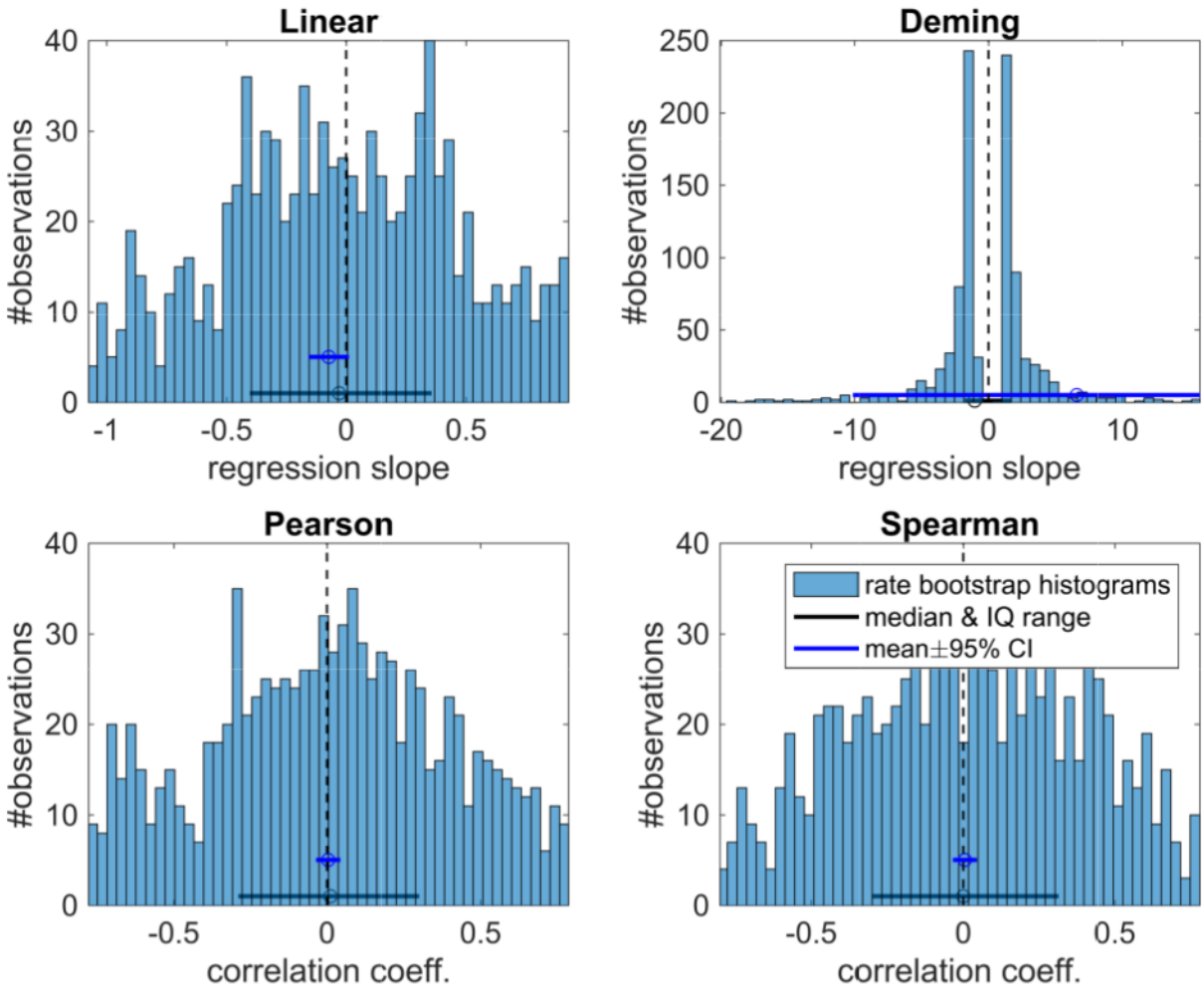


Supplementary Figure 7. Virulence of *C. glabrata* wild-type ancestral, small and regular colony size variants in *G. mellonella* larvae (second replicate study). Survival of groups of 20 *G. mellonella* wax moth larvae injected with 2.5 x 10^6 CFU/larva per strain over 7-day incubation at 37°C. (a) 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).

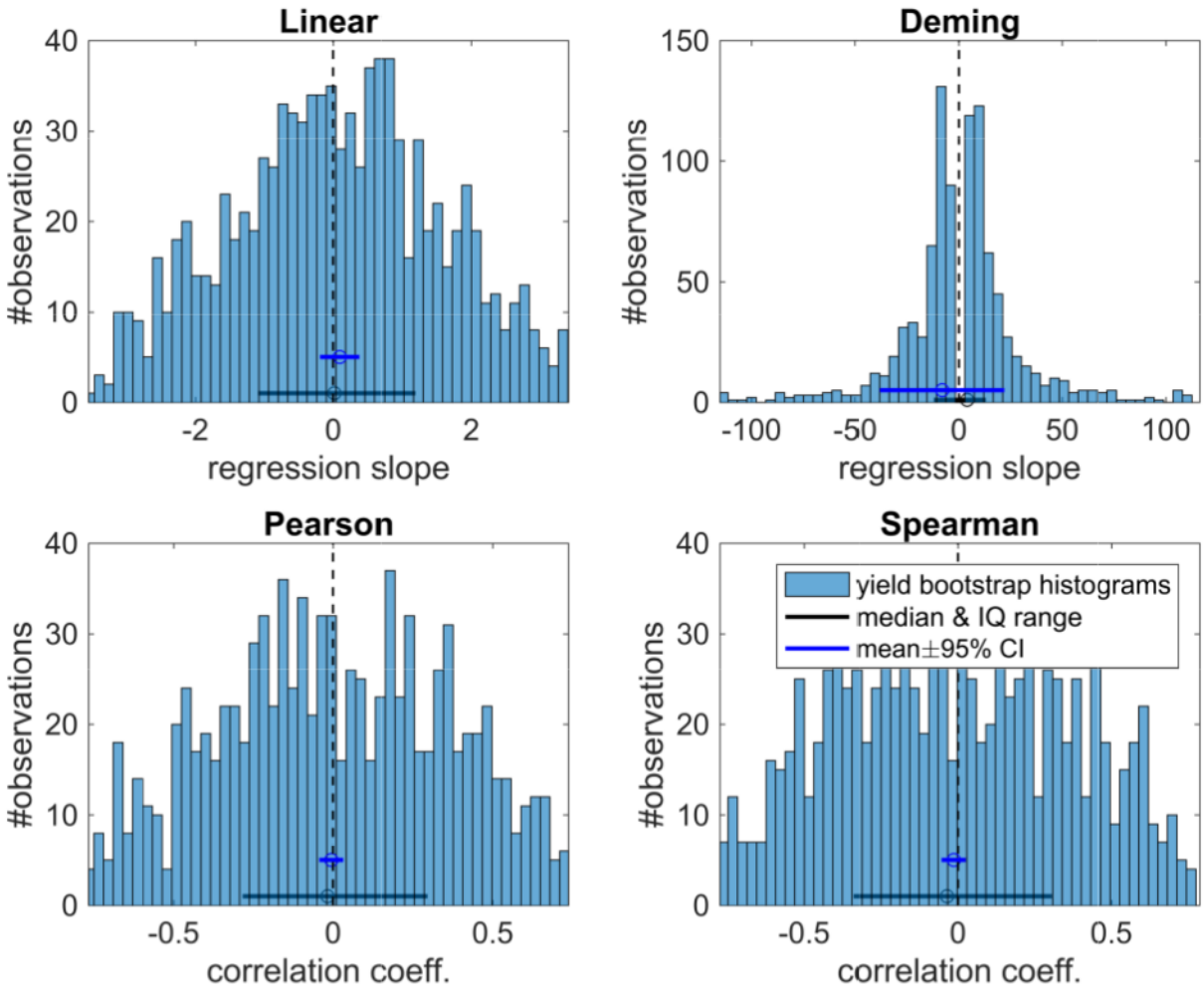


Supplementary Figure 8. Virulence of *C. glabrata* wild-type ancestral, small and regular colony size variants in *G. mellonella* larvae (third replicate study). Survival of groups of 20 *G. mellonella* wax moth larvae injected with 2.5 x 10^6 CFU/larva per strain over 7-day incubation at 37°C. (a) WT = 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)

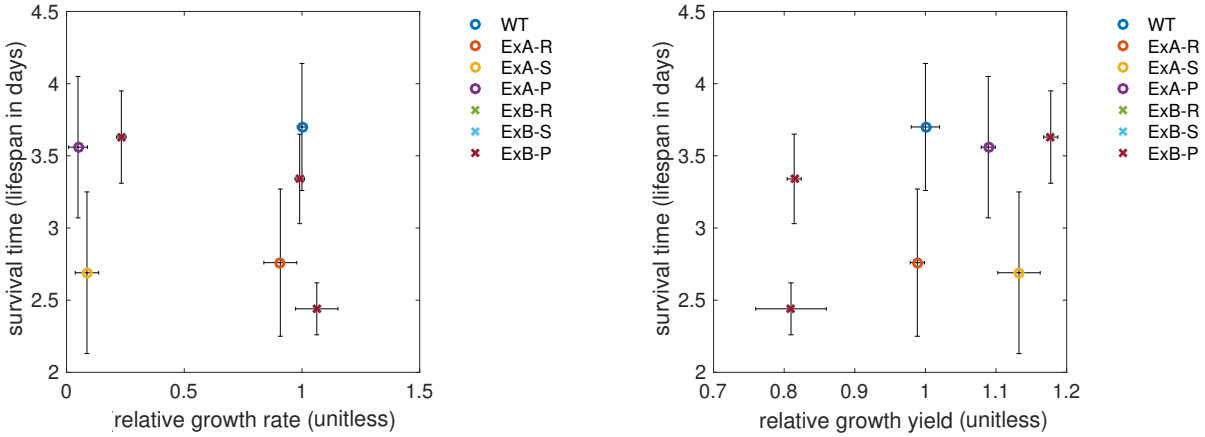

Linear
Deming
Pearson
Spearman
#observations
regression slope
correlation coeff.
rate bootstrap histograms
median & IQ range
mean±95% CI

(b)

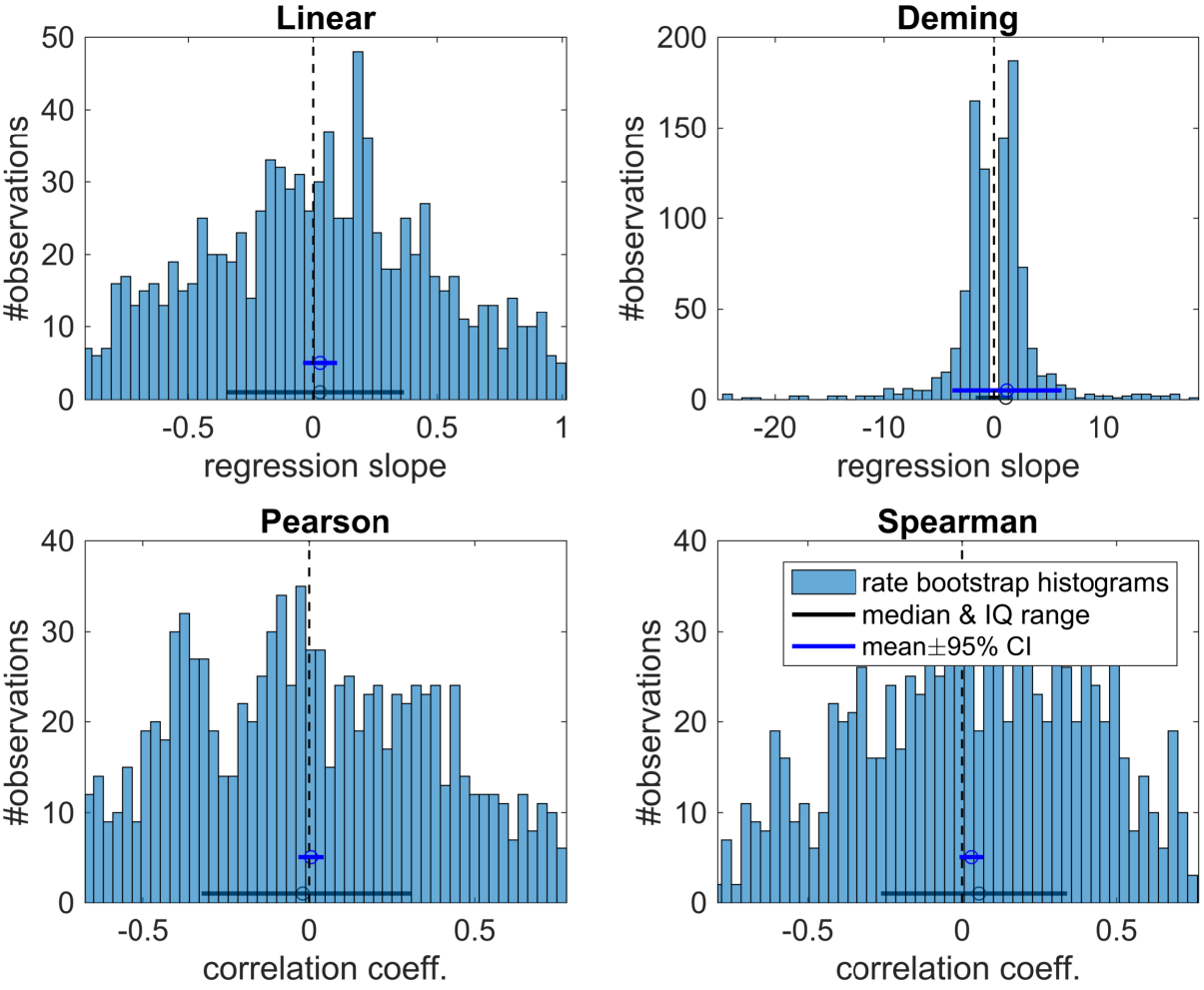


Supplementary Figure 9. Growth rate, growth yield and virulence (first replicate study). Analysis of data presented in Figure 4 where growth traits were plotted against larval survival time for all strains from Experiments A and B, including regular colony variants and small colony variants before and 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).

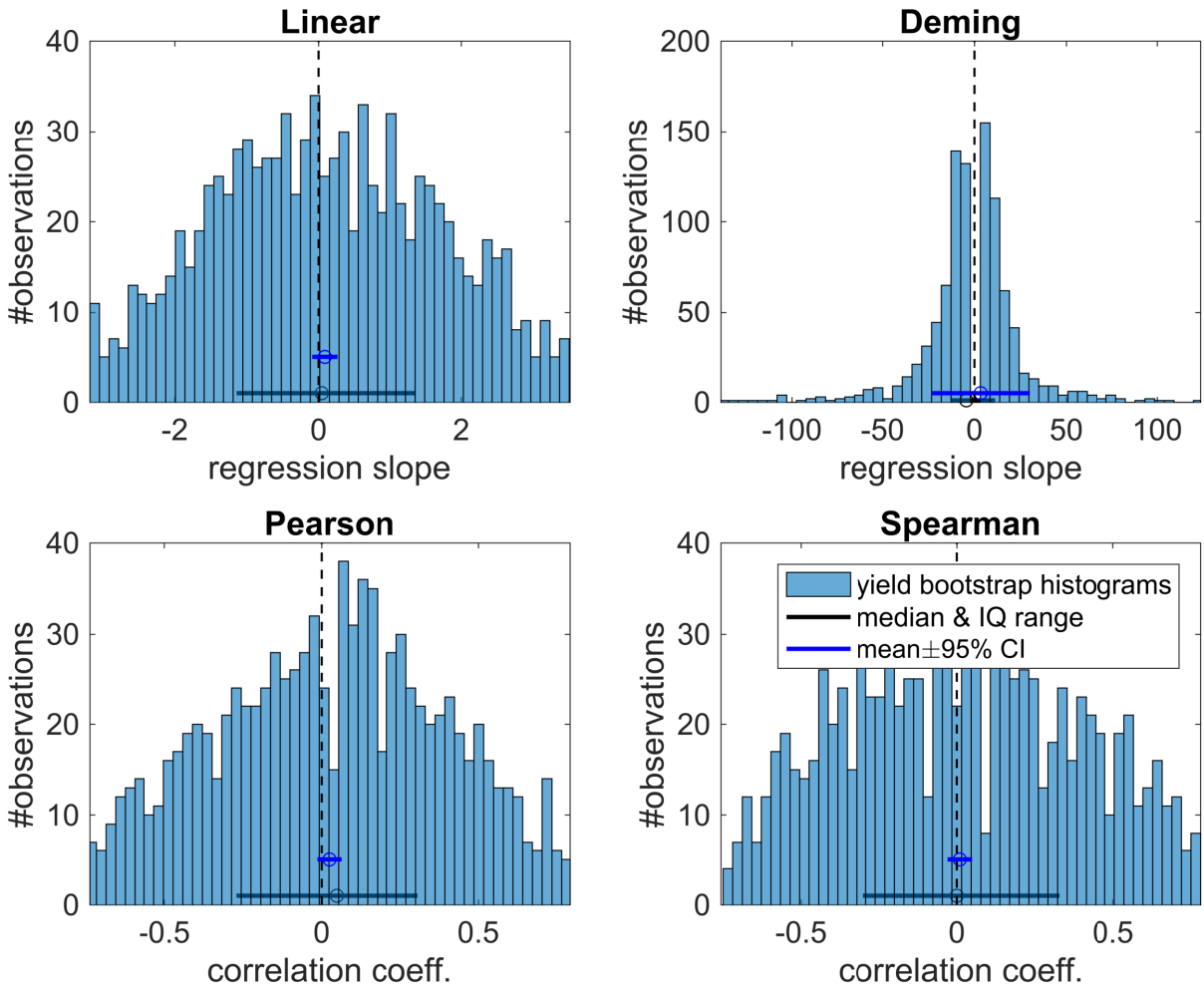
(a)


survival time (lifespan in days)
relative growth rate (unitless)
relative growth yield (unitless)
WT
ExA-R
ExA-S
ExA-P
ExB-R
ExB-S
ExB-P

(b)

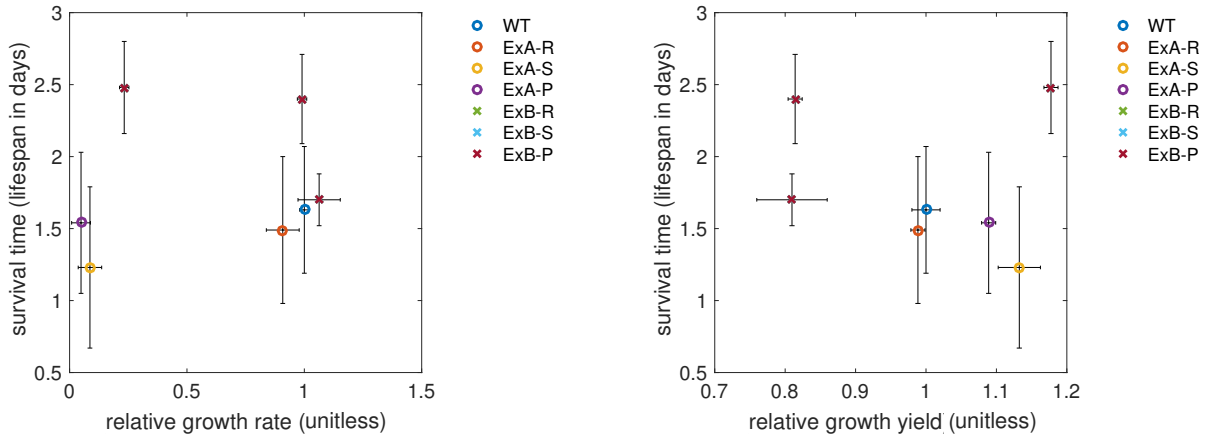

Linear
Deming
Pearson
Spearman
#observations
regression slope
correlation coeff.
rate bootstrap histograms
median & IQ range
mean±95% CI

(c)

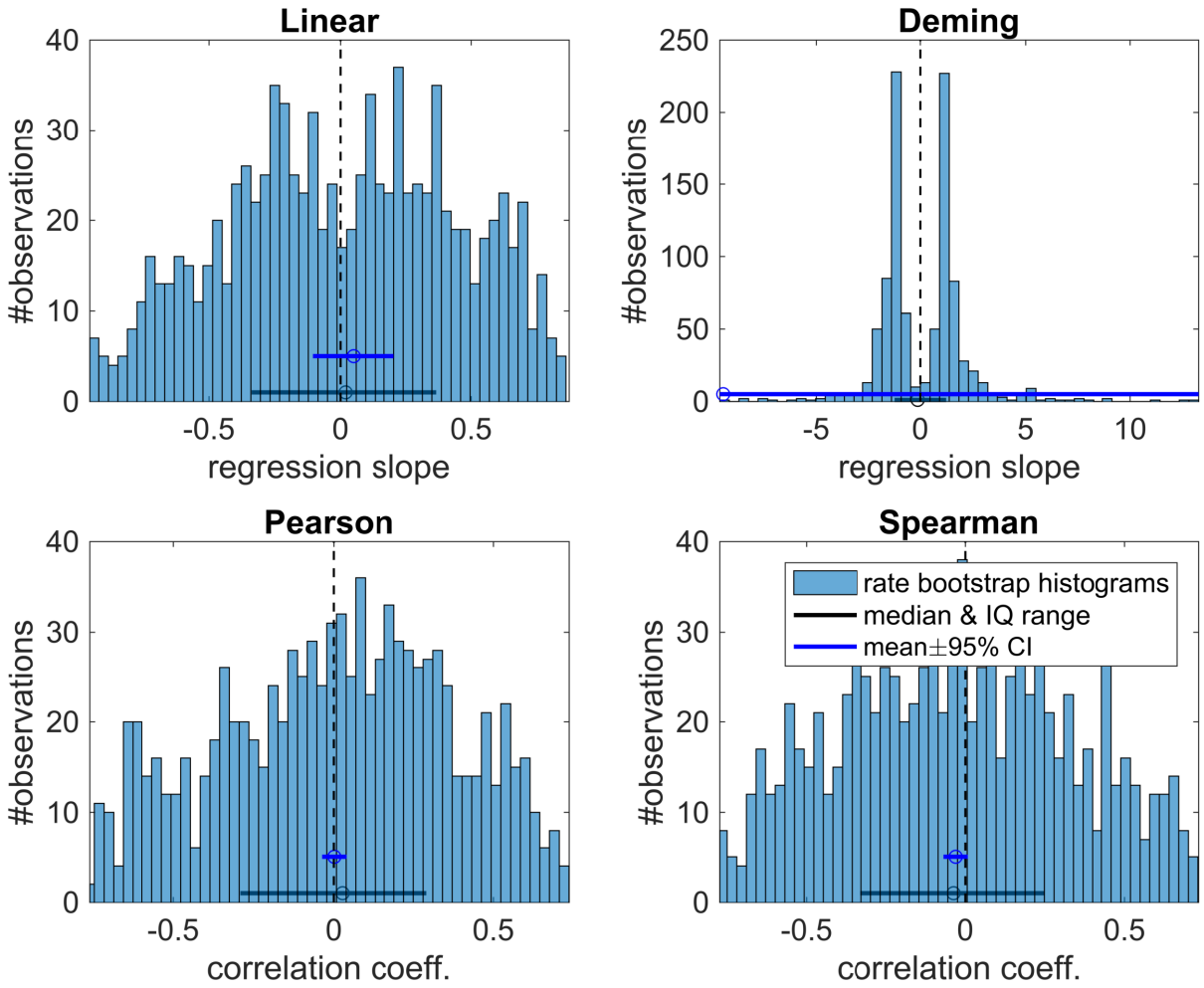


Supplementary Figure 10. Growth rate, growth yield and virulence (second replicate study). (a) Growth traits are plotted for all strains from Experiments A and B, including regular colony variants and small colony variants before and after passaging. Data is combined from Figures 1a, b, 2 and Supplementary 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).

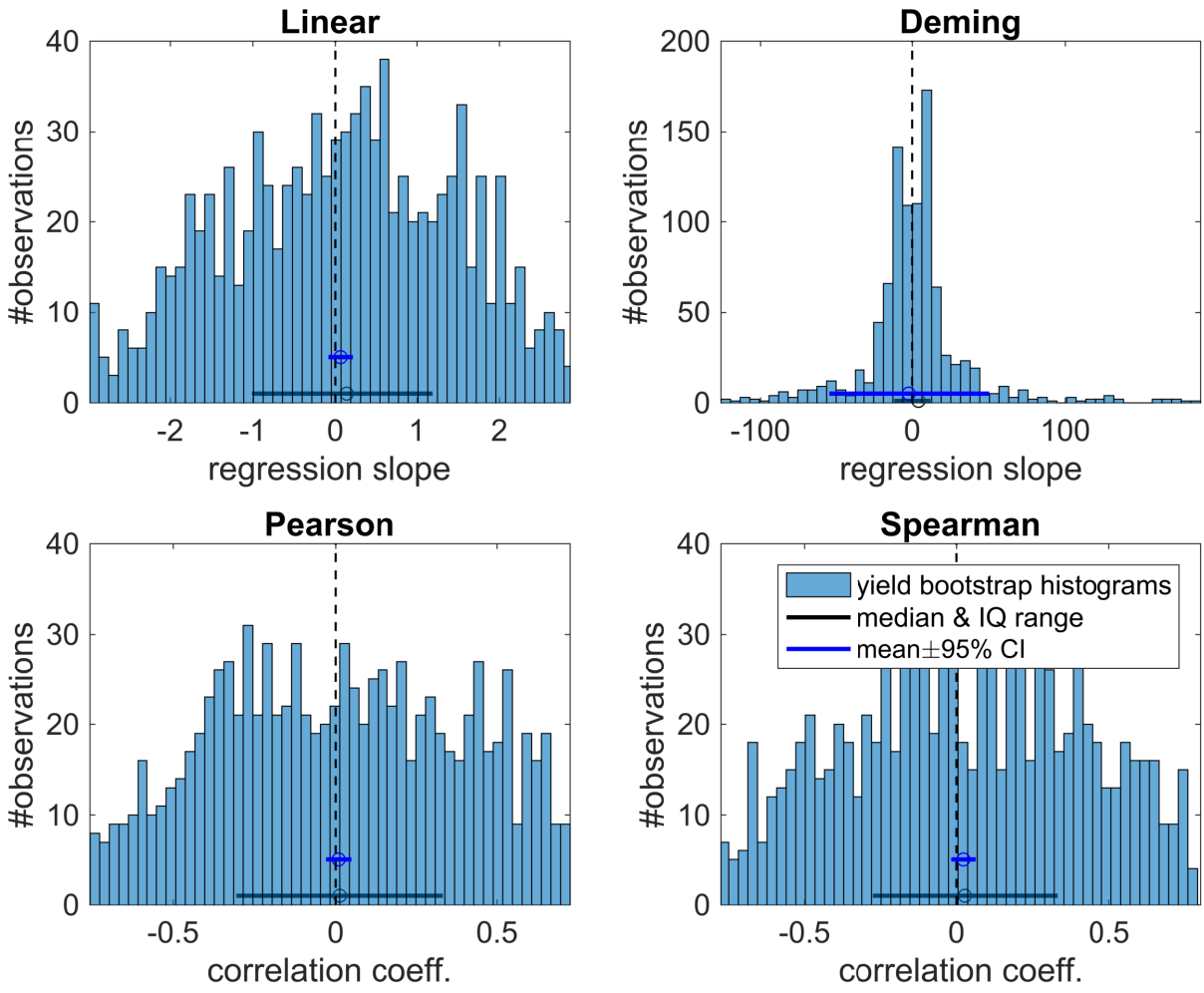
(a)


survival time (lifespan in days)
relative growth rate (unitless)
relative growth yield (unitless)
WT
ExA-R
ExA-S
ExA-P
ExB-R
ExB-S
ExB-P

(b)


Linear
Deming
Pearson
Spearman
#observations
regression slope
correlation coeff.
rate bootstrap histograms
median & IQ range
mean±95% CI

(c)



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).