Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in *Escherichia coli*

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Supplementary online material

Contents

Supplementary Methods 2
Supplementary Results 4
Supplementary Tables 11
Supplementary Figures 17
References 42

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Supplementary Methods

Defining stressor concentration in each environment: The selection of the maximum stressor pressure that can be applied without inhibiting growth was done as follows. For adaptation in osmotic stress, we used 0.3M NaCl concentration as it has been previously used to induce osmotic stress in *E. coli* (Gunasekera et al, 2008). For estimating the maximum acidic stress we can apply to the cells, we tested several M9 media with reduced pH and found that pH of ≤ 4.0 did not reproducibly support growth of *E. coli* MG1655 in serial passages over 48h. Adaptation to low pH was performed at a starting pH of 5.5 by preparing M9 medium and adjusting the pH to 5.5 followed by sterilization using a 0.22μm Millipore filter unit. Adaptation to oxidative stress was achieved by adding H_2O_2 to the growth medium. We tested several concentrations of H_2O_2 in the range of 10 to 500μM in serial transfers for 48 hours. A concentration of 100μM H_2O_2 represented the uppermost limit for reliable growth and was used in all oxidative stress experiments. Since H_2O_2 is known to be unstable in diluted solutions a 0.6% stock solution in HQ-H_2O was prepared, sterilized by a 0.22μM syringe filter and stored at 4°C. This stock solution was used for 7 days and then replaced by a new stock solution. A previous study showed that a concentration of 0.8% (v/v) of n-butanol does not lead to significant cell death and growth arrest in *E. coli* DH1(Rutherford et al, 2010). We tested concentrations in the range of 0.4 to 1.2% (v/v) and M9 salts medium supplemented with 0.6% n-butanol was used for adaptive evolution experiments of *E. coli* MG1655 growth.

Serial passages and laboratory evolution: We selected a daily dilution ratio of 1:500 so that cells remain under exponential growth without experiencing a daily stationary phase, both to maximize the number of generations per day and minimize the effect of stationary phase adaptation in our results. We observed significant fitness advantage in the case of media adaptation, as cells adapted to the control environment (G500 strains) had significantly faster growth than the ancestral strains (Fig. S7). After 500 generation, growth curves were obtained for all strain/environment combinations (Fig. S8) and maximum growth rates (μ_{max}) where obtained (Table S-III). For calculating the maximum growth rate the following formula was used:

$$\mu_{max} = (X_{t2} - X_{t1})/(t_2 - t_1),$$

where X_t is cell density estimated by optical density at time t, and in the interval chosen growth follows a strictly exponential pattern (Fig. S9). For a more accurate measure of adaptation, competition assays

with high reproducibility (Table S-IV and Fig. S10) were performed for various strain pairs and under all environmental contexts (Table S-V and Fig. S11- S15), which enabled us to rank the adapted populations based on their relative fitness in the respective environments (Lenski et al, 1998). Although both methods yielded similar results, there were some cases where max growth rate was not a good predictor of competitive fitness as in the case of oxidative stress.

Estimated fitness relative to the G500 strain: Because 4 biological replicates with two replicates for the MG1655 strain and 2 replicates for the Δ*lacZ* strain were cultivated per growth condition, direct competition assays by blue/white screening were possible (see methods section in the main text for details). These direct competitions of strains under various stressors were used to estimate the relative fitness of the populations (Table S-V lists relative fitness values measured by direct competition assays). Indirect estimates were obtained in the following cases: (1) "P500 *vs.* G500" under osmotic stress from "B500 *vs.* P500" and "B500 *vs.* G500" assays; (2) "H500 *vs.* G500" under osmotic stress from "H500 *vs.* P500", "B500 *vs.* P500" and "B500 *vs.* G500" assays; (3) "B500 *vs.* G500" under butanol stress from "B500 *vs.* O500" and "O500 *vs.* G500" assays; (4) "H500 *vs.* G500" under butanol stress from "P500 *vs.* H500" and "P500 *vs.* G500" assays; (5) "B500 *vs.* G500" under oxidative stress from "H500 *vs.* B500" and "H500 *vs.* G500" assays; (6) "P500 *vs.* G500" under oxidative stress from "H500 *vs.* P500" and "H500 *vs.* G500" assays; (7) "O500 *vs.* G500" under acidic stress from "O500 *vs.* P500" and "P500 *vs.* G500" assays. This estimation assumes growth independence of two competing stains, i.e. that each stain's growth curve is invariant with respect to the presence of any other competing strain in the same medium.

Darwinian fitness (*W*) was used as a proxy for fitness of the stress evolved populations in all environments. The method of calculating Darwinian fitness *W* is described by Lenski et al. (http://myxo.css.msu.edu/ecoli/srvsrf.html):

$$mX = \ln[Xt_1/Xt_0]/day$$

$$W = mA / mB$$

As such, mX is the realized Malthusian parameter for population X with Xt_1 being the estimated cell density of population X at time 1 and Xt_0 being the estimated cell density of population X at time 0. *W* is then defined as the ratio of the Malthusian parameters of 2 competing populations A and B with mA and mB being calculated as described for an arbitrary population mX.

Genome resequencing: For genome resequencing the best biological replicate per stress adaptation experiment was chosen (Fig. S16, Table S-V). In all cases the selected replicate represents *E. coli* MG1655 background. From each selected biological replicate four individual colonies were picked from a LB agar plate. A growth curve was recorded for each of these clones in order to select the best performing clone for genome sequencing (clone rankings are shown in Table S-VI).

SNP and indel detection: SNPs and variances between sequenced strains and the reference *E. coli* genome were obtained for each strain by analyzing both: (i) shotgun reads mapped to the reference genome by *BWA* (Li & Durbin, 2010) and *SAMtools* (Li et al, 2009), and (ii) *de novo* assembly followed by the alignment to the reference genome (the protocol is described in the methods section of the paper). Variances which are found in all strains are attributed to the ancestral strain; unique variances are the result of the independent evolution of the strains.

Supplementary Results

Overlap of transcriptional profiles in different stress conditions: Based on available literature that reports the changes of gene expression patterns of *E. coli* under several stress conditions (Aertsen et al, 2004; Bianchi & Baneyx, 1999; Choi et al, 2003; Dürrschmid et al, 2008; Gill et al, 2000; Gunasekera et al, 2008; Richmond et al, 1999; Rutherford et al, 2010; Weber et al, 2005; White-Ziegler et al, 2008; Zheng et al, 2001), we compiled a list of overlapping gene/protein expression changes under the following stressors: high and low temperature, low pH, osmo-, *n*-butanol, oxidative (H_2O_2), high hydrostatic pressure, stationary phase and recombinant protein production stress. The Venn diagrams in Fig. S1 illustrate the overlap of the differentially expressed genes.

Neutrality of the *lacZ* deletion in *E. coli* MG1655: The two *E. coli* strains that were used in our study were the *E. coli* MG1655 strain and a MG1655 derivative that lacked the β-galactosidase gene (MG1655 Δ*lacZ*). The inclusion of the *ΔlacZ* strain allowed us to perform direct competition assays between the adapted and ancestral lineages with the addition of X-Gal and IPTG. Growth tests on M9 medium and LB medium indicated that the MG1655 Δ*lacZ* showed no altered growth behavior when compared to the MG1655 strain (Fig. S2). In addition, direct competition assays (Fig. S3) over 48h (4 biological

replicates) demonstrated the neutrality of the Δ*lacZ* mutation in all environments that are relevant to this study (Table S-I and Fig. S4).

Adaptation under a single environmental stressor: *E. coli* strains were evolved in M9 salts medium with glucose as the sole carbon source and the following stressors: osmotic stress (0.3M NaCl, O500 strain), acidic stress (pH 5.5, P500 strain), oxidative stress (100μM H_2O_2, H500 strain), *n*-butanol stress (0.6% *n*-butanol, B500 strain), and control (no-stress, G500). The OD_{600} of each culture was measured each day before the daily transfers to ensure that the estimated 9 generations per day were reached (Table S-II and Fig. S5). The addition of NaCl, H_2O_2 and *n*-butanol did not influence the pH of M9 medium giving an initial pH of 7.0 ± 0.1 (± min/max). However, since pH fluctuations in the medium were not compensated by buffering substances, over a 24h cultivation the pH would decrease to 6.0 ± 0.1 (min/max) in all environments except the acidic stress environment, where the pH would reach 3.9 ± 0.1 from an initial value of 5.5. Final pH values after 24h of cultivation did not change in any of the evolved strains as compared with the ancestral strains.

Population variation during adaptation: Recent reports in short-term laboratory evolution show high phenotypic heterogeneity in the adapted population (Wang et al, 2010). To test the degree of phenotypic variance and to guide further experimentation, we analyzed 3 individual clones from each biological replicate (12 in total) from cultures adapted in the control (no-stress) and osmotic conditions. We observed significant clone-to-clone variation after 500 generations (Fig. S6). Consequently, our competition assays were performed on a population level to avoid clonal outliers during evaluation of fitness potential.

Direct competition assays in different environments: Only two significant inconsistencies were observed in the direct competition assay dataset (see Table S-V for the relative fitness values): (1) under oxidative stress both H500 and O500 outcompete the B500 strains with close relative fitness values of 1.37±0.06 and 1.44±0.03, respectively, while same strains H500 and O500 have significantly different fitness relative to the G500 strain (1.433±0.07 and 0.98±0.04, respectively); (2) under acidic stress P500, H500 and G500 strains have close fitness based on "P500 *vs.* H500" and "H500 *vs.* G500" assays (1.08±0.02 and 0.98±0.04, respectively), however P500 significantly outcompetes the G500 strain in a direct competition with the relative fitness of 1.20±0.02.

Genome re-sequencing: From reads mapped to the reference *E. coli* MG1655 genome (GenBank accession no. U00096.2), 14 SNPs were identified in five strains at positions where the minus consensus quality score (-FQ) was above 38 (which is a Phred-scaled probability of all reads not being the same, while being different from the reference; it corresponds to the p-value of $1.6 \cdot 10^{-4}$). No positions with a variance relative to the reference genome had a minus consensus quality score between 0 and 38. In order to find longer indels, five *de novo* assembled genomes were aligned with the reference MG1655, and all variants were collected. Out of 1,255 positions with possible variances one deletion and thee insertions were identified. The rest of the positions with variances were found in the highly repetitive regions of the *E. coli* genome (transposases, prophages, insertion elements, tRNA-s, and rRNA-s), where the *de novo* assembly fails (see Table S-VIII for the full list of the breaks in the *de novo* assembly).

Ancestral genome: We sequenced five closely related genomes and we were able to distinguish mutations specific to only one strain, from mutations present in all genomes. The later mutations were attributed to the ancestral strain. Reconstructed ancestral genome has seven novel genetic variations (Table S-X) relative to the reference MG1655 genome (Hayashi et al, 2006), six of which were confirmed independently (Freddolino et al, 2012). We identified an additional IS2 insertion in the *yeaJ* locus.

Detection of gene amplification: Gene duplications were detected similarly to RNA-Seq analysis by mapping pair-end Illumina reads (obtained during the shoot gun sequencing of stress evolved strains) to the transcriptome of the reference *E. coli* K12 MG1655 strain. For each gene, counts of mapped reads were collected. While the coverage varies significantly across the genome, the coverage for each individual gene is much more constant. Only 6% of genes have the maximum to average coverage ratio above 1.25. Top amplified genes were found in two regions of the *E. coli* genome (see Fig. S17): (i) fragment from 606,179 to 614,717 with 12-fold amplification in O500 strain evolved under osmotic stress (7 genes including *fepA* and *fes* genes from enterobactin-iron transport and hydrolysis system); and (ii) a fragment from 3,617,200 to 3,764,250 with two-fold amplification in P500 strain evolved in low pH environment (114 genes including all 13 genes from the Acid Fitness Island (Mates et al, 2007): *gadW*, *gadX*, *gadA*, and others).

Types of fixed mutations: In all resequenced strains we found 4 fixed mutations per strain after 500 generations, in total 14 nucleotide polymorphisms (SNPs), 3 transposon insertions, one 85bp deletion, and two genomic regions were amplified (8kbp and 147kbp long), see Table I in the main text. This distribution of mutations correlates well with other adaptive laboratory evolution (ALE) experiments (Barrick et al, 2009; Charusanti et al, 2010; Christopher et al, 2006; Conrad et al, 2009; Conrad et al, 2011; Goodarzi et al, 2010; Goodarzi et al, 2009; Kishimoto et al, 2010; Lee et al, 2011; Woods et al, 2006). SNPs are generally the most common types of mutations observed in ALE: 70% of all mutations in our study and on average 61% of mutations found in recent bacterial evolution experiments (Conrad et al, 2011). The most common substitutions observed in the past are C→T and G→A corresponding to 43% of all observed SNPs (Conrad et al, 2011). In our study the top substitutions are: G→T, C→T, and A→C observed 4, 3, and 3 times out of 14, respectively (Table S-X). Interestingly, the majority of SNPs are substitutions of purines with smaller nucleotides, pyrimidines (11 out of 14 SNPs). Ten top genes with most mutations in ALE experiments are summarized in (Conrad et al, 2011) with *rpoB* being the most mutated gene under high-temperature conditions (Tenaillon et al, 2012). In our study we found that two of these genes mutated in evolved strains: *rpoB* (in G500, O500, B500, and H500) and *pykF* in G500. The *rpoB* gene is the β subunit of RNA polymerase (RNAP), which is responsible for the majority of catalytic functions carried by RNAP (Jin & Gross, 1989). Previously, mutations in *rpoB* gene where observed in ALE experiments at gene positions 1,685, 4,006, and 3,724 in *E. coli* strains evolved in, glycerol minimal media (Christopher et al, 2006), lactate minimal media (Conrad et al, 2009), and glucose minimal media followed by the deletion of *pgi* gene (Charusanti et al, 2010), respectively. In our experiments, four out of five evolved strains carry an *rpoB* mutation. O500 and P500 strains have two long amplifications: 8kbp and 147kbp, respectively. 12-fold amplification in the O500 strain includes *fepA* gene, and a 2-fold amplification in the P500 strain (147kbp long 3,617,200-3,764,300, located between highly homologous *rhsB* and *rhsA* genes) contains an Acid Fitness Island, a group of 13 genes related to the bacterial acid resistance (Mates et al, 2007). A similar region (140kbp long, approximately 3,620,000-3,760,000) was duplicated in one of the strains evolved in a minimal lactate media (Conrad et al, 2009).

Adaptation and the rate of fixation: As mutations are mostly accumulated during DNA replication, cumulative number of cell divisions (CCD) is a good proxy for the adaptation timescales. Recently it was estimated that *E. coli* populations evolving under the growth rate selection pressure in the minimal

M9 media and a three-carbon compound (glucose, glycerol, L-lactate, or L-1,2-PDO) achieve a (first) stable phenotype in about $10^{11.2}$ CCDs with approximately 2 to 8 fixed mutations (Lee et al, 2011). In the presence of mutagen (NTG) the rate of mutation accumulation increases by about two orders of magnitude, however the number of CCDs to reach a stable phenotype is decreased only by about a factor of three. In our study, in addition to the minimal media and glucose carbon source, evolving populations were exposed to various abiotic stresses. This increases the strength of the selection pressure, which now can more rapidly detect small-scale mutations (Roth, 2010) and could potentially result in a faster adaptation with the number of required CCDs to reach a stable phenotype even lower, than in the presence of a mutagen.

Timeline of adaptation: After 500 generations each strain accumulated about 4 mutations and had approximately $10^{10.6}$ to $10^{10.9}$ CCDs, as the growth rate was the lowest under the pH stress and the highest in the M9 salt glucose media (using the estimation method described in (Lee et al, 2011)). First, stable phenotypes under *n*-butanol and osmotic stress emerged after about 290 generations (32 days, see Fig. S5), which is equivalent to about $10^{10.5}$ and $10^{10.4}$ CCDs, respectively. Interestingly, it is at least 5 times faster than it was observed for *E. coli* adaptation in M9 carbon source media with no additional abiotic stress (Lee et al, 2011). Populations evolved under the oxidative or acidic stresses accumulated 4 mutations each after 500 generations ($10^{10.6}$ and $10^{10.8}$ CCDs, respectively). While growth rates have been increased in only a subset of the conditions, evolved strains clearly out-compete the reference G500 strain under the stress conditions they were adapting for (Fig. 2 in the main text).

Gene expression analysis: Illumina reads for RNA-Seq libraries were mapped to the reference *Escherichia coli* strain K12 substrain MG1655 genome by BWA toolkit (Li & Durbin, 2010). Differentially expressed genes were identified by processing raw counts of mapped reads with *edgeR* library. Gene ontology analysis was performed by *goseq R* package. Differentially expressed (DE) genes in all stress evolved strains were found relative to the expression levels in the reference G500 strain using the *edgeR R* package. Genes with BH (Benjamini and Hochberg) adjusted p-values below 0.05 threshold were selected as DE genes. Genes from the amplified regions in O500 (12-fold) and P500 (two-fold) strains are significantly over-expressed relative to the reference: average log Fold Change for concentrations is +3.6 and +0.8 (both with p-values$<10^{-20}$), respectively. The RNA-seq dataset can be obtained from the GEO repository, record no. GSE39926. The expression level of genes that were

differential regulated and where also involved in identified mutations were further validated by qRT-PCR with a high correlation coefficient ($\rho \approx 0.9$). More specifically, the RNA-Seq, qRT-PCR for each gene and strain are the following: O500, *fepA* up-regulation 10x for RNA-Seq, 9-11x for qPCR, *proV* down-regulation 2.7x for RNA-Seq, 3x for qPCR, B500, *fepA* up-regulation x2.3 RNA-Seq, 2.9x qPCR, *marC* up-regulation 1.2x, 1.1 for qPCR, H500 *katG* up-regulation 2.8x for RNA-Seq, 3.1x qPCR, P500 *gadX* up-regulation 1.6x for RNA-Seq, 1.4x qRT-PCR. Full list of genes DE in at least one of the strains and sorted by the expression patterns is shown in Table S-XI.

Correlation between iron- and acid-response pathways: Data suggest that there is a link between iron- and acid- stress response pathways in *E. coli* (Figure S18) similarly to one found in *Shigella flexneri* (Oglesby et al, 2005). This inhibitory link between *ryhB* and *evgA* is not present in current *E. coli* pathways databases, however recent studies suggest a high correlation between iron-regulation and acid response pathways in *E. coli* (Zhu et al, 2002), *H. pylori* (Bijlsma et al, 2002); *S. typhimurium* (Hall & Foster, 1996), and *C. glutamicum* (Jakob et al, 2007).

Maximum stress tolerance: To investigate whether adaptation under a specific stress increases also the tolerance to higher stress concentrations, we grew ancestral and two sets of *n*-butanol and high salt adapted strains (adapted for 500 and 1000 generations respectively) to various *n*-butanol or NaCl concentrations. Adapted strains were found to have an increased tolerance to higher stress concentrations, and the same result was obtained with osmotic stress-adapted cells (Suppl. Fig. S19 and S20). Interestingly, when the same experiment was performed under LB media (instead of M9 under which the cells had adapted), especially for the salt adapted populations, we observed an inverse relationship between the time adapted under stress/M9 media and maximum stress tolerance: ancestral cells had a three-fold difference in OD600 than O500 cells, which in turn had a two-fold difference than O1000 cells (Suppl. Fig. S20).

Gene regulatory network: Gene regulatory network regulatory network of *E. coli* was reconstructed from the data available in EcoCyc (Keseler et al, 2011) and RegulonDB (Gama-Castro et al, 2011) databases as a directed graph with nodes being genes and positive, negative, or zero weights of edges for activation, inhibition, or un-defined regulation, respectively (Figure S21A). The known network contains 1724 nodes/genes (about 41% of all *E. coli* genes) and 4153 regulatory links; 200 nodes act as regulators to at least one other gene, while other genes are only being regulated (terminal nodes). The

sub-network of pathways involved in regulation of genes differentially expressed in stress evolved strains contains 82 regulators (41% of all regulators in the known gene regulatory network) and includes key stress response genes, such as: *gadX*, *gadY*, *gadW*, *ydeO* (acid stress response), *soxS* (superoxide response), etc. Figure S21B shows the sub-network of nodes connected to genes differentially expressed in at least one of the stress-evolved strains.

Supplementary Tables

Table S-I. Average fitness values of *E. coli* MG1655 Δ*lacZ* compared with *E. coli* MG1655 in different M9+glucose (0.4% w/v) media. Observed Darwinian fitness (W) data represent average values of 4 biological replicates ± standard error of the mean (SEM). 95% CI confidence level, *p* Student's T test.

Growth medium	W	95% CI	*p*
M9+glucose	0.992 ± 0.004	0.007	0.200
0.3M NaCl	0.998 ± 0.007	0.030	0.805
pH 5.5	1.011 ± 0.002	0.004	0.092
100mM H_2O_2	0.997 ± 0.002	0.004	0.946
0.6% *n*-butanol	0.990 ± 0.007	0.013	0.703

Table S-II. Based on daily OD600 measurements the following generations were obtained for the 5 different stress conditions (average ± standard deviation).

Stress condition	Generations
M9+glucose	8.9 ± 0.4
0.3M NaCl	8.9 ± 0.4
pH 5.5	9.0 ± 0.3
100mM H_2O_2	9.0 ± 0.4
0.6% *n*-butanol	8.9 ± 0.5

Table S-III. Maximum growth rates μ_{max} [h^{-1}] of the evolved strains on all growth media used in this study. Values present the average of 4 biological and 2 technical replicates ± the standard error of the mean.

	M9+glucose	0.3M NaCl	pH 5.5	0.6% *n*-butanol	100μM H_2O_2
Ancestor	0.498 ± 0.005	0.300 ± 0.007	0.441 ± 0.006	0.325 ± 0.005	0.470 ± 0.009
G500	0.618 ± 0.006	0.353 ± 0.004	0.462 ± 0.008	0.317 ± 0.008	0.535 ± 0.008
O500	0.610 ± 0.017	0.384 ± 0.004	0.396 ± 0.007	0.358 ± 0.013	0.540 ± 0.006
P500	0.565 ± 0.005	0.313 ± 0.012	0.492 ± 0.032	0.353 ± 0.005	0.493 ± 0.016
H500	0.536 ± 0.018	0.336 ± 0.009	0.405 ± 0.029	0.314 ± 0.012	0.512 ± 0.007
B500	0.568 ± 0.005	0.344 ± 0.008	0.461 ± 0.032	0.385 ± 0.001	0.496 ± 0.013

Table S-IV. Observed Darwinian Fitness (W) for the independent repetitions of competition assays

	Strains; medium	Fitness	SD	95% CI
Assay I	O500, G500; 0.3M NaCl	1.405 ± 0.043	0.168	0.082
Assay II	O500, G500; 0.3M NaCl	1.533 ± 0.076	0.153	0.075
Assay I	P500, G500; pH 5.5	1.379 ± 0.080	0.320	0.157
Assay II	P500, G500; pH 5.5	1.340 ± 0.063	0.252	0.123

Table S-V. Results of direct competition assays of evolved *E. coli* populations on different M9 based growth media (population A *vs.* population B). Observed Darwinian fitness (W) data represent average values of 4 biological replicates ± standard error of the mean (SEM). *p-value* is calculated through student's T test. Asterisk (*) indicates that fitness values were measured after 24h of competition (instead of 48h).

Population A	Population B	Medium	Fitness (W)	*p-value*
O500	G500	M9+glucose	1.001 ± 0.017	0.947
G500	H500	M9+glucose	1.044 ± 0.011	0.018
G500	P500	M9+glucose	1.043 ± 0.011	0.054
B500	P500	M9+glucose	1.062 ± 0.011	0.008
G500	B500	M9+glucose	0.957 ± 0.013	0.178
O500	G500	0.3M NaCl	1.386 ± 0.049	$2.0 \cdot 10^{-11}$
O500	B500	0.3M NaCl	0.989 ± 0.021	0.694
B500	P500	0.3M NaCl	1.257 ± 0.017	$3.4 \cdot 10^{-06}$
B500	G500	0.3M NaCl	1.514 ± 0.048	$1.1 \cdot 10^{-09}$
H500	P500	0.3M NaCl	0.975 ± 0.028	0.466
P500	G500	pH 5.5	1.203 ± 0.023	$6.7 \cdot 10^{-07}$
H500	G500	pH 5.5	0.981 ± 0.043	0.660
P500	H500	pH 5.5	1.081 ± 0.019	0.063
B500	P500	pH 5.5	1.314 ± 0.019	$5.6 \cdot 10^{-08}$
O500	P500	pH 5.5	1.283 ± 0.026	$3.7 \cdot 10^{-04}$
B500	O500	pH 5.5	1.042 ± 0.046*	0.365
B500	G500	pH 5.5	1.416 ± 0.052	$1.9 \cdot 10^{-05}$
H500	G500	0.1mM H_2O_2	1.334 ± 0.065	$1.7 \cdot 10^{-06}$
O500	G500	0.1mM H_2O_2	0.980 ± 0.041	0.968
O500	B500	0.1mM H_2O_2	1.436 ± 0.030	$3.1 \cdot 10^{-07}$
H500	P500	0.1mM H_2O_2	1.259 ± 0.044	$8.0 \cdot 10^{-07}$
H500	B500	0.1mM H_2O_2	1.368 ± 0.055	$4.9 \cdot 10^{-05}$
B500	O500	0.6% *n*-butanol	1.441 ± 0.018	$2.2 \cdot 10^{-09}$
O500	G500	0.6% *n*-butanol	1.096 ± 0.015	$4.9 \cdot 10^{-04}$
P500	G500	0.6% *n*-butanol	1.058 ± 0.015	0.016
P500	H500	0.6% *n*-butanol	1.077 ± 0.010	$6.0 \cdot 10^{-04}$

Table S-VI. The 4 biological replicates of each stress adaptation experiments were ranked, based on their maximum growth rate M - MG1655 , L – Δ*lacZ.*

	O500 on 0.3M NaCl	G500 on M9+gluc	P500 on pH 5.5	H500 on 0.1mM H_2O_2	B500 on 0.6% *n*-butanol
Best	O500 M1	G500 M1	P500 M1	H500 M1	B500 L2
	O500 M2	G500 M2	P500 L1	H500 L1	B500 M1
	O500 L1	G500 L1	P500 L2	H500 L2	B500 M2
Weakest	O500 L2	G500 L2	P500 M2	H500 M2	B500 L1

Table S-VII. Statistics of resequencing of stress evolved *E. coli* strains on the Illumina Genome Analyzer GAII.

	G500	**H500**	**B500**	**O500**	**P500**
Average coverage	202	133	145	204	174
Preprocessing reads (SGA)					
Total reads	10,771,690	8,689,028	9,284,150	12,453,740	10,626,890
Reads kept, %	94.98%	94.53%	93.80%	94.55%	93.66%
Assembly (IBDA)					
Number of contigs	374	320	314	413	367
Total contig length	4,587,626	4,583,389	4,581,406	4,591,496	4,586,267
Coverage	184	148	158	212	180
Maximum contig size	221,635	221,637	221,637	221,640	166,315
Average contig size	12,266	14,323	14,590	11,117	12,496
N50	71,354	71,351	60,902	61,589	60,904
Insert size	232	264	279	242	241
Scaffolding (SSPACE)					
Number of scaffolds	213	190	170	256	196
Total scaffold length	4,580,683	4,578,455	4,576,452	4,583,802	4,579,036
Maximum scaffold size	223,146	289,736	256,037	311,779	223,146
Average scaffold size	21,505	24,097	26,920	17,905	23,362
N50	135,569	129,257	135,158	105,887	107,098

Table S-VIII. (Please see the supplementary file Table_S-VIII.xlsx) List of the genome assembly breaks in five sequenced strains. Positions are shown for the reference *E. coli* K12 MG1655 genome.

Table S-IX. Mutations of the ancestral *E. coli* MG1655 strain used in the current study compared with the reference sequence for *E. coli* MG1655.

Mutation type	Genomic reference postition	Change, Gap/insert length	Affected genetic locus
Ins	257,908	155	IS1 insertion in *crl* (DNA-binding transcriptional regulator)
SNP	547,694	A→G	*ylbE_1*, hypothetical protein, Glu -> Gly
Ins	547,832	G→GG	upstream of *ylbE_2*
Ins	1,871,054	406	IS2 insertion in *yeaJ* (predicted diguanylate cyclase)
Ins	2,171,385	CC→CCCC	*gatC* (galactitol-specific enzyme IIC component of PTS)
Del	3,558,477	CG→C	*glpR* (transcriptional repressor)
SNP	3,957,957	C→T	Intergenic region *ppiC* and *yifO* (hypothetical protein), 93bp upstream of *ppiC* transcriptional start

Table S-X. Types of single nucleotide substitutions found in five stress evolved strains. Nucleotides substituted in the ancestry strain are the column labels, substituting nucleotides (in the evolved strains) are the row labels.

		Purines		Pyrimidines	
		G→	A→	C→	T→
Purines	→G	–	1		
	→A		–	1	1
Pyrimidines	→C		3	–	
	→T	4	1	3	–

Table S-XI. (Please see the supplementary file Table_S-XI.xlsx) Expression levels of genes differentially expressed in at least one of the stress-evolved strains under the osmotic stress (with p-value <0.05); $\log_2$ (Fold Change relative to G500 strain) and p-values are shown for all strains. Expression levels of genes significantly differentially expressed in at least one of the strains are highlighted with color: over-expressed values are marked with green, under-expressed values are marked with red, and over-expressed values for amplified genes are marked with yellow.

Table S-XII. Results of direct competition assays of evolved B500 and B500 repair mutants on different M9 based growth media (population A relative to population B). *acrA*, *fepA*, *marX*, and *rpoB* indicate B500 clone with respective mutations repaired and introduced chloramphenicol resistance. Observed Darwinian fitness (W) data represent average values of 2 independent competitions (3 independent competition plates were averaged for each competition in each experiment) ± standard error of the mean (SEM). *p-value* is calculated through student's T test.

Population A	Population B	Medium	Fitness (W)
acrA	B500	M9+glucose	0.958 ± 0.016
fepA	B500	M9+glucose	0.850 ± 0.005
marC	B500	M9+glucose	0.956 ± 0.036
rpoB	B500	M9+glucose	0.959 ± 0.013
B500	G500	M9+glucose	1.130 ± 0.019
acrA	B500	0.3M NaCl	1.035 ± 0.024
fepA	B500	0.3M NaCl	0.721 ± 0.026
marC	B500	0.3M NaCl	0.946 ± 0.060
rpoB	B500	0.3M NaCl	0.977 ± 0.005
B500	G500	0.3M NaCl	1.412 ± 0.028
acrA	B500	0.6% *n*-butanol	0.927 ± 0.007
fepA	B500	0.6% *n*-butanol	0.864 ± 0.015
marC	B500	0.6% *n*-butanol	0.321 ± 0.243
rpoB	B500	0.6% *n*-butanol	0.927 ± 0.007
B500	G500	0.6% *n*-butanol	1.674 ± 0.119
acrA	B500	0.1mM H_2O_2	0.977 ± 0.020
fepA	B500	0.1mM H_2O_2	0.897 ± 0.005
marC	B500	0.1mM H_2O_2	0.961 ± 0.022
rpoB	B500	0.1mM H_2O_2	1.042 ± 0.008
B500	G500	0.1mM H_2O_2	1.103 ± 0.030
acrA	B500	pH 5.5	0.888 ± 0.031
fepA	B500	pH 5.5	0.703 ± 0.001
marC	B500	pH 5.5	0.845 ± 0.032
rpoB	B500	pH 5.5	0.965 ± 0.029
B500	G500	pH 5.5	2.464 ± 0.246
MG1655 + chloramphenicol resistance	MG1655	M9+glucose	0.957 ± 0.004

Supplementary Figures

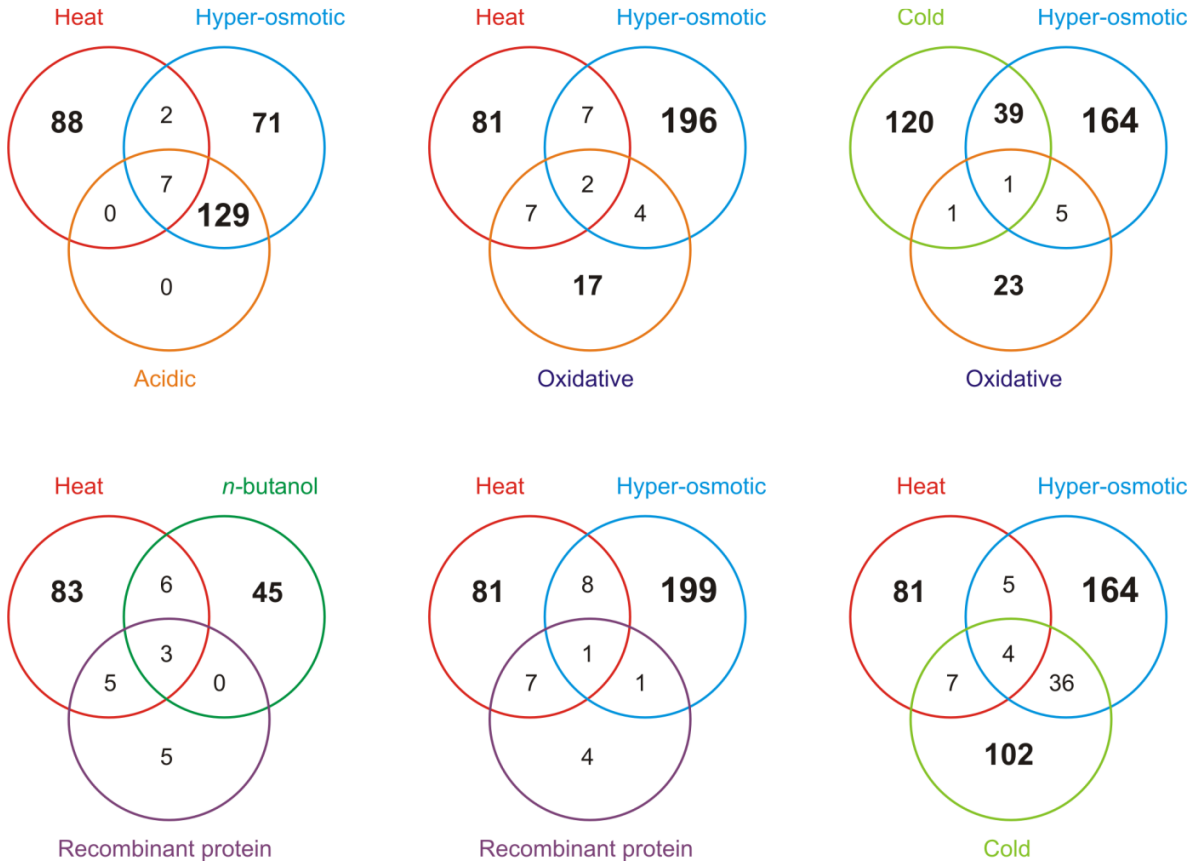


Figure S1. Overlap of differentially expressed genes during stress. Venn diagrams illustrating the overlap of stress response mechanisms during short-term exposure (from several minutes to 10 generations) to different stress conditions: heat (high temperature); cold (low temperature); hyper-osmotic stress; oxidative stress (hydrogen peroxide-induced oxidative stress); *n*-butanol induced stress; recombinant protein-induced stress.

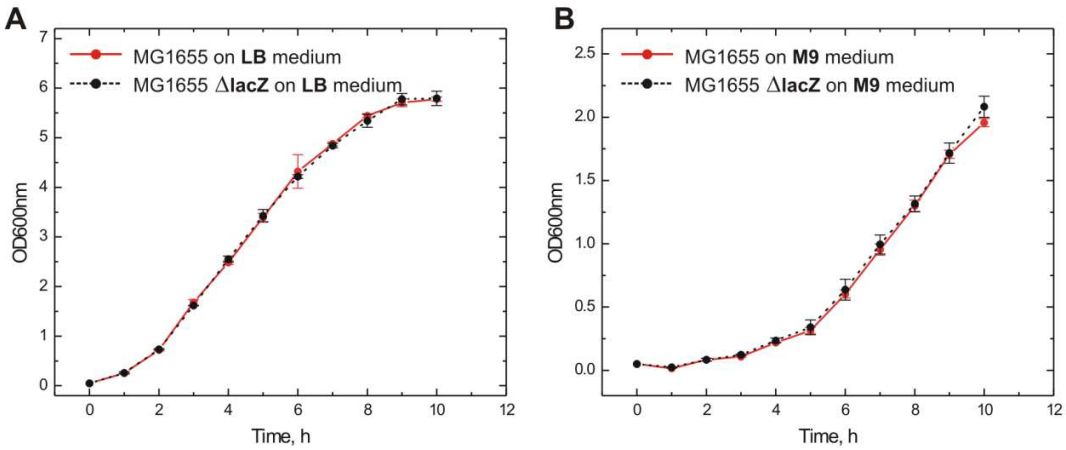


Figure S2. Growth curves of the MG1655 and ΔlacZ mutant strains. Growth curves of *E. coli* MG1655 and MG1655 Δ*lacZ* on LB medium (A) and M9 medium (B). Red solid lines – *E. coli* MG1655, dashed black lines – *E. coli* MG1655 Δ*lacZ*. Data represent averages of 3 replicates. Error bars show the standard deviation.

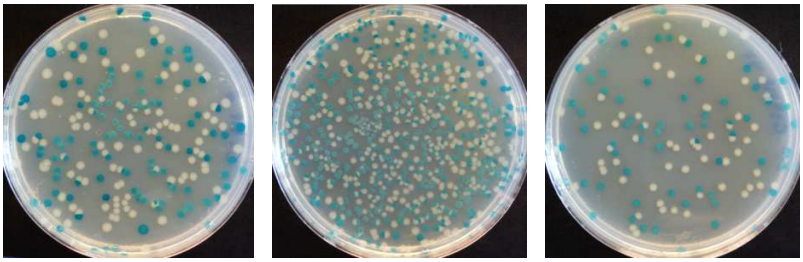


Figure S3. Snapshots of a competition assay depicting Δ*lacZ* neutrality. Example of a direct competition assay between *E. coli* MG1655 and *E. coli* MG1655 Δ*lacZ* on M9+glucose medium. Samples taken at 0, 24 and 48h of growth. Ratio (MG1655/Δ*lacZ*) at 0, 24 and 48h was 97/103, 425/364 and 70/62, respectively.

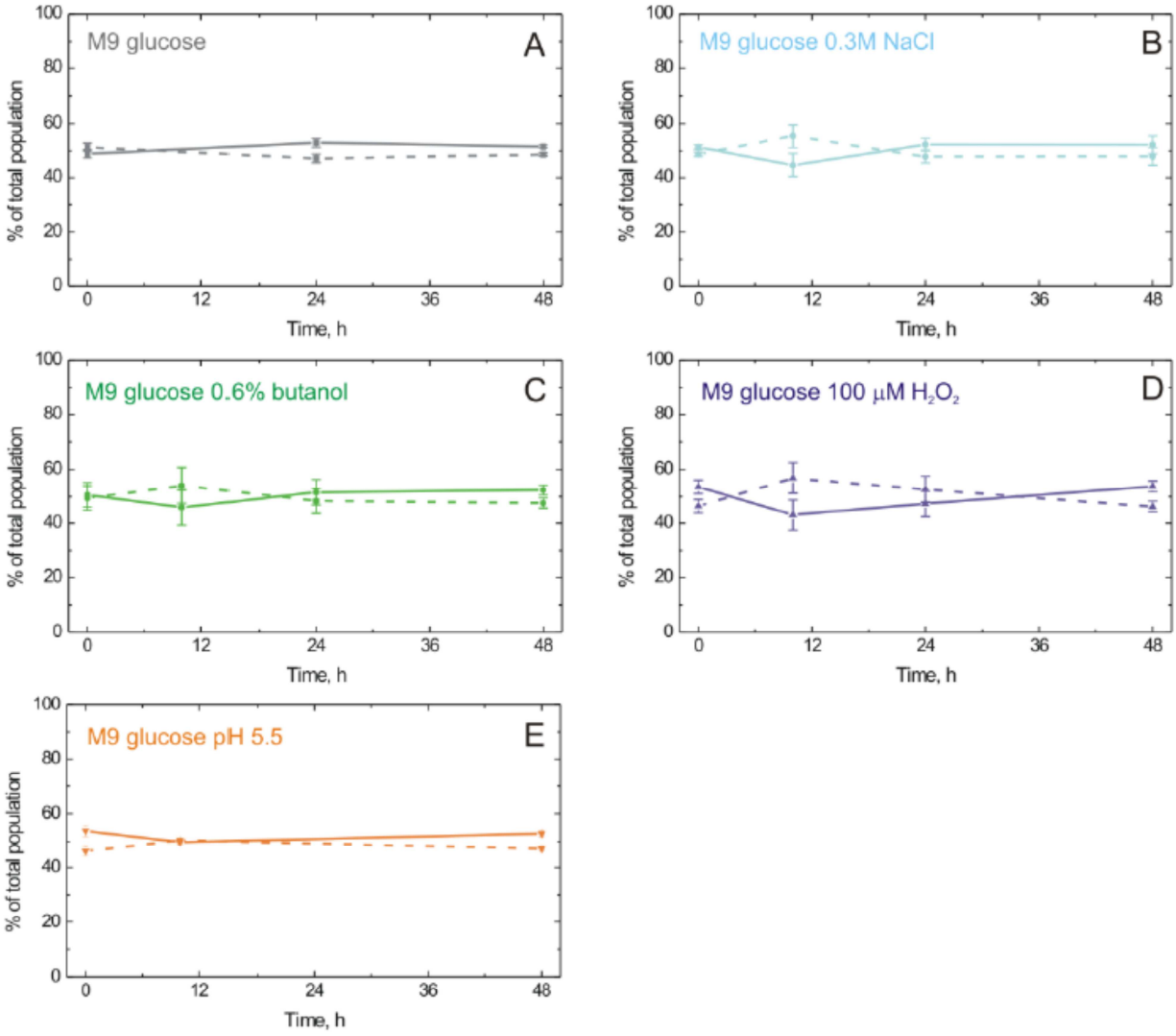


Figure S4. Competition assays illustrate Δ*lacZ* neutrality in all environmental conditions. Competition of ancestral *E. coli* strains in different M9 media. Solid lines: *E. coli* MG1655, dashed lines: *E. coli* MG1655 Δ*lacZ*. (A) No stress; (B) hyper-osmotic stress (0.3M NaCl); (C) *n*-butanol stress (0.6% *n*-butanol); (D) oxidative stress (100mM H_2O_2); (E) acidic stress (pH 5.5). The medium is always M9 salt with glucose as a sole carbon source. Values represent averages of 4 biological replicates ± standard error of the mean.

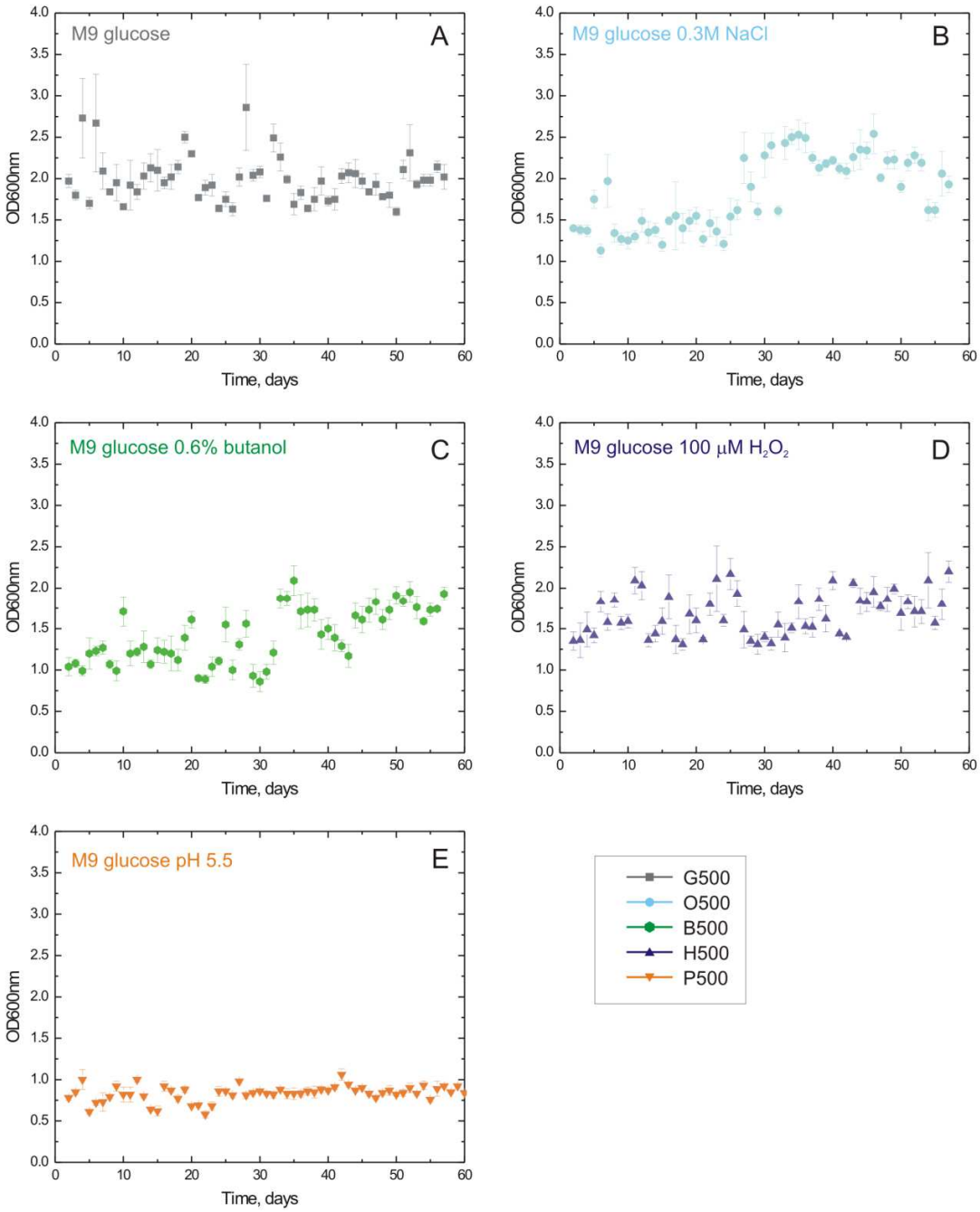


Figure S5. Cell density fluctuations for all environments over the course of the experiment. Daily measurements of OD600 during the serial passages until 500 generations were reached. Data represent the average OD600 values of 4 replicate cultures (2x *E. coli* MG1655 and 2x *E. coli* MG1655 Δ*lacZ*). Error bars represent the standard error of the mean. (A) No stress; (B) hyper-osmotic stress (0.3M NaCl); (C) *n*-butanol stress (0.6% *n*-butanol); (D) oxidative stress (100mM H_2O_2); (E) acidic stress (pH 5.5). The medium is always M9 salt with glucose as a sole carbon source.

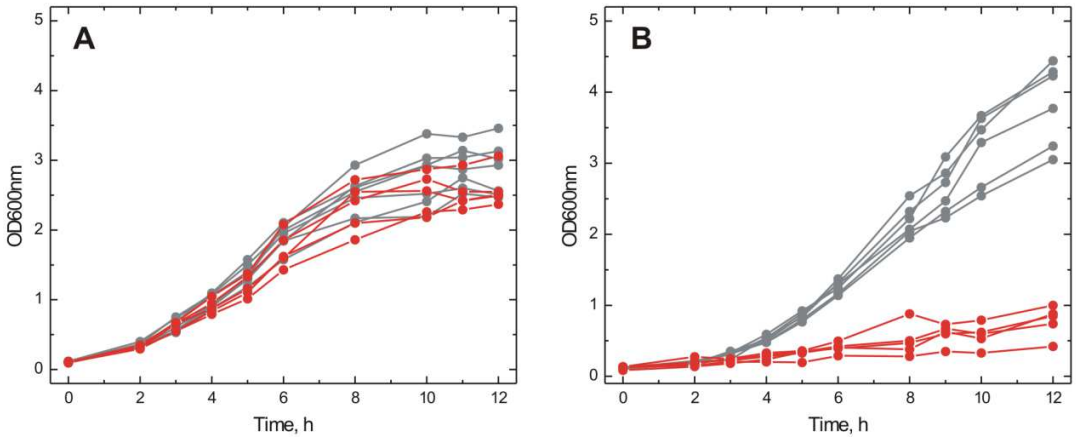


Figure S6. Phenotypic variance in a G500 strain under osmotic stress. Growth curves of 12 individual clones of *E. coli* strains (3 clones per biological replicate) that were adapted on M9 glucose medium for 500 generations (G500 strain). Growth was recorded for: (A) no stress; (B) hyper-osmotic stress (0.3M NaCl). The medium is always M9 salt with glucose as a sole carbon source. Clones highlighted in red show evolutionary trade-offs towards hyper-osmotic stress resistance.

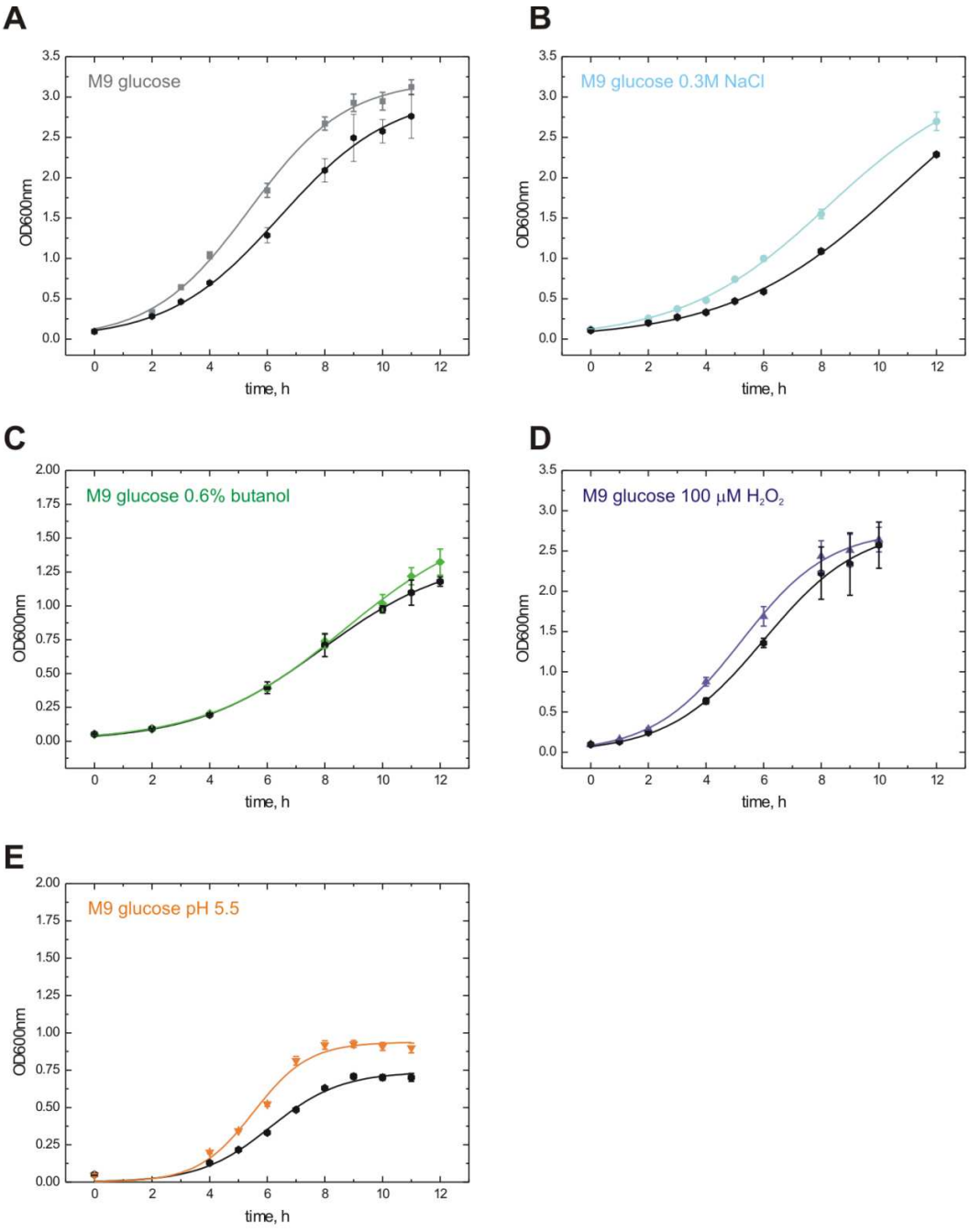


Figure S7. Growth rate difference between the G500 and the ancestral strains. Growth curves of *E. coli* strains (4 biological replicates) evolved on M9 glucose medium and of the ancestral *E. coli* strains. (A) No stress; (B) hyper-osmotic stress (0.3M NaCl); (C) *n*-butanol stress (0.6% *n*-butanol); (D) oxidative stress (100mM H_2O_2); (E) acidic stress (pH 5.5). The medium is always M9 salt with glucose as a sole carbon source. Experimental data were fitted by sigmoidal logistic curve fitting.

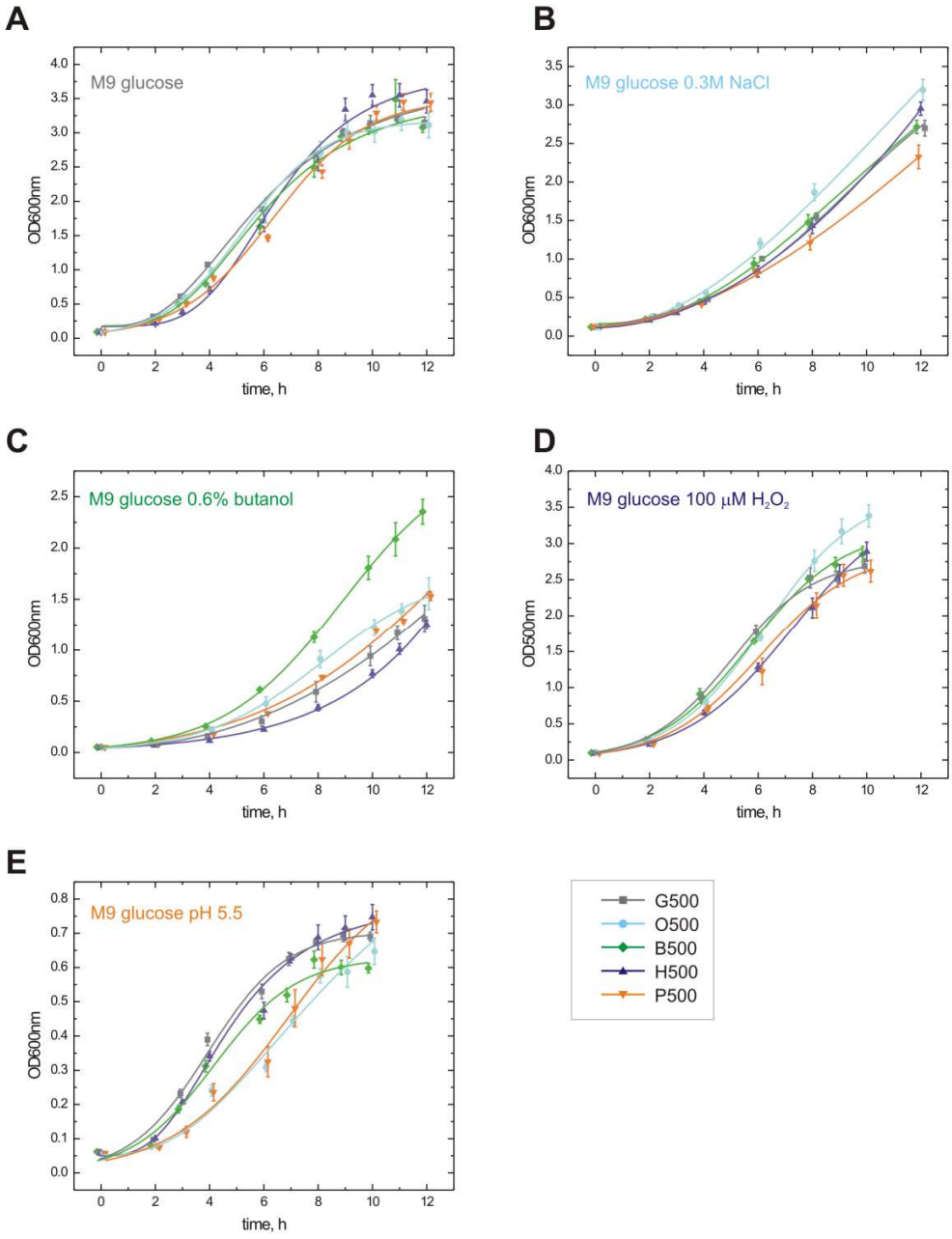


Figure S8. Cross-strain comparison of growth characteristics in all environmental conditions. Growth curves of the evolved strains on the media used in this study. (A) No stress; (B) hyper-osmotic stress (0.3M NaCl); (C) *n*-butanol stress (0.6% *n*-butanol); (D) oxidative stress (100mM H_2O_2); (E) acidic stress (pH 5.5). The medium is always M9 salt with glucose as a sole carbon source. All growth curves were started at OD600 = 0.1 except for tests under the acidic stress (pH 5.5) where sinitial OD600 was 0.05. Data represent sigmoidal logistic curve fittings of data points obtained from 4 biological and 2 technical replicates.

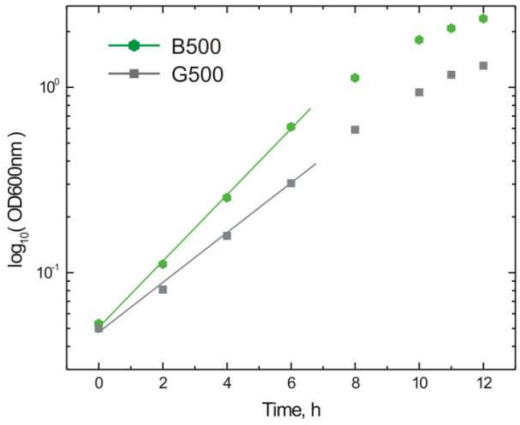


Figure S9. Calculation of maximum growth rate μ_{max}. Growth of cells evolved under *n*-butanol stress (0.6% *n*-butanol) and under no stress tested under *n*-butanol stress (0.6% *n*-butanol). The medium is always M9 salt with glucose as a sole carbon source. OD600nm as a measure of the cell growth was plotted on a logarithmic scale to highlight strictly exponential growth phase, which was selected for calculation of μ_{max}.

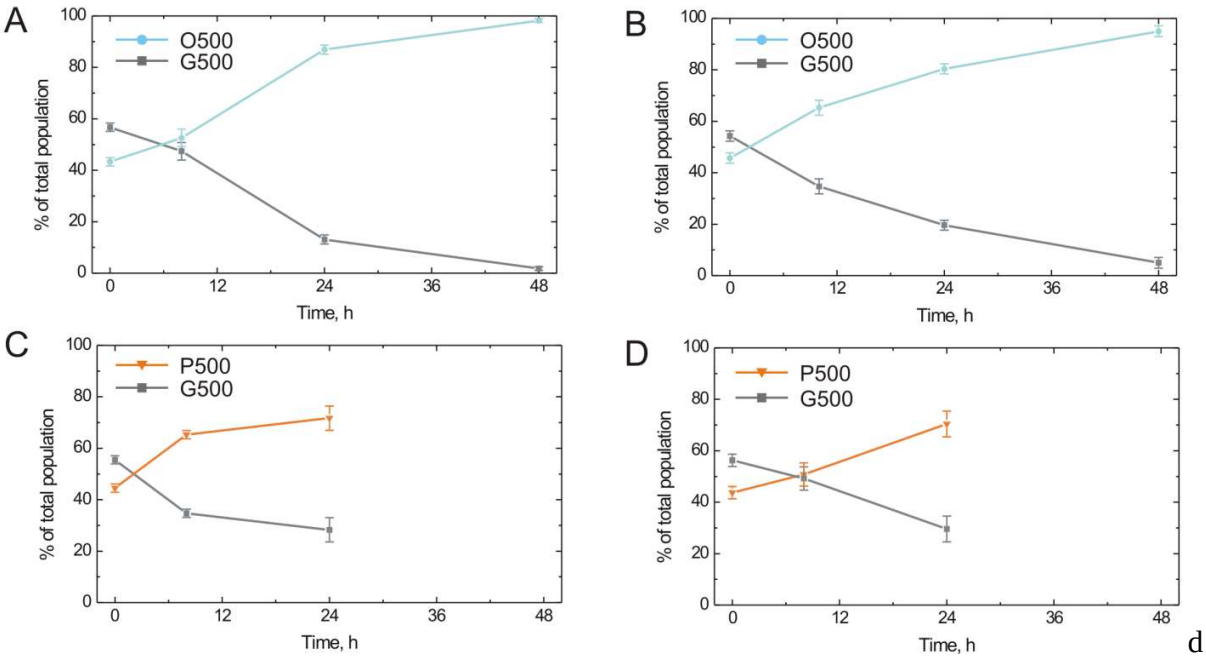
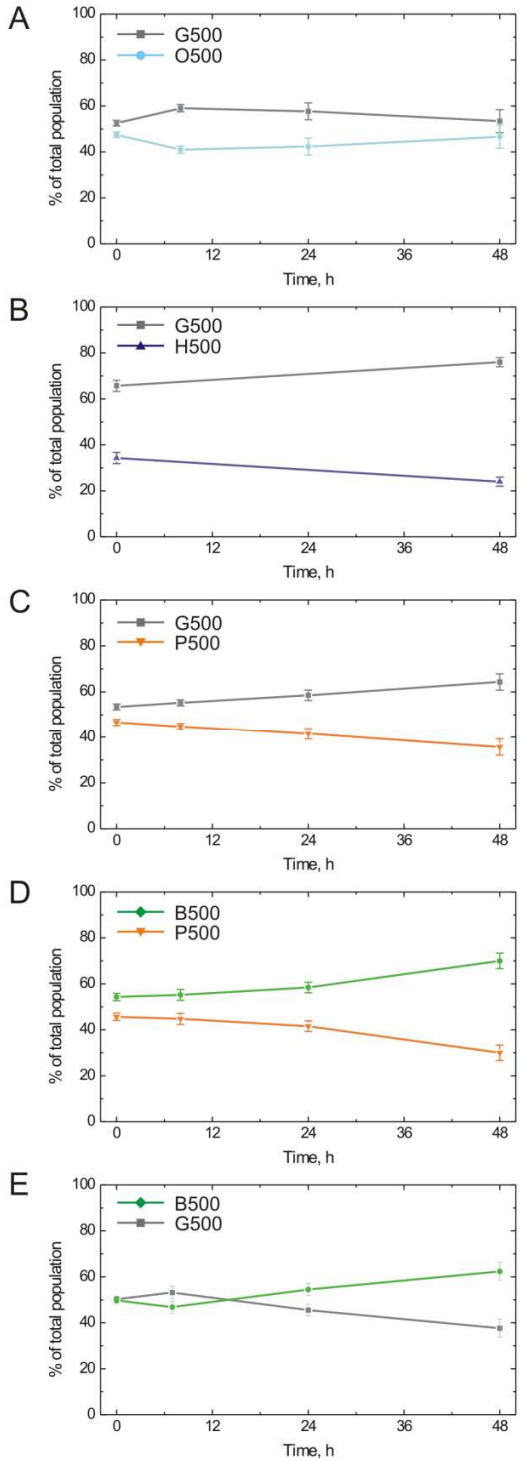


Figure S10. **Reproducibility of Competition Assays.** Replication of Competition assays for the (A,B) G500 and O500 under hyper-osmotic stress (0.3M NaCl) and (C,D) G500 and P500 under acidic stress (pH 5.5). The medium is always M9 salt with glucose as a sole carbon source. In order to evaluate to reproducibility of the direct competition assays we performed 2 independent replicates of 2 pairs for competition. For each of the assays cells were taken independently from cryo and competed for a total of 48h. For each independent competition 4 biological and 2 technical replicates were performed.



Strain A	Strain B	W
O500 ΔlacZ #1	G500 MG1655 #1	1.13
O500 ΔlacZ #2	G500 MG1655 #1	1.01
O500 ΔlacZ #1	G500 MG1655 #2	1.07
O500 ΔlacZ #2	G500 MG1655 #2	0.90
O500 MG1655 #1	G500 ΔlacZ #1	0.96
O500 MG1655 #2	G500 ΔlacZ #2	0.98
O500 MG1655 #1	G500 ΔlacZ #1	0.96
O500 MG1655 #2	G500 ΔlacZ #2	0.99

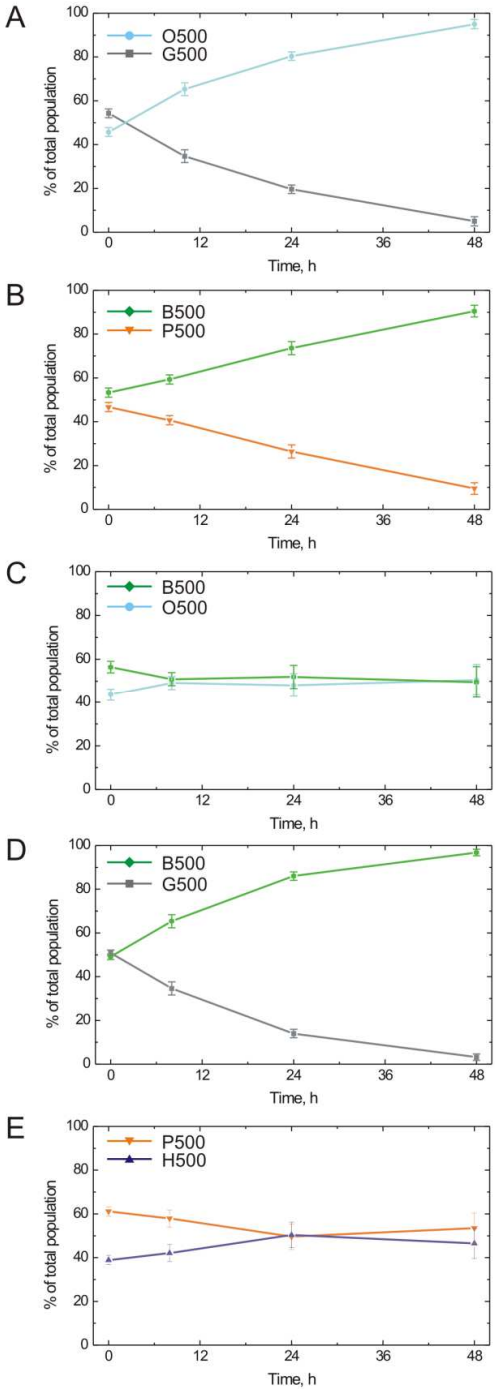
Strain A	Strain B	W
H500 ΔlacZ #1	G500 MG1655 #1	0.91
H500 ΔlacZ #2	G500 MG1655 #1	0.94
H500 ΔlacZ #1	G500 MG1655 #2	1.02
H500 ΔlacZ #2	G500 MG1655 #2	0.99
H500 MG1655 #1	G500 ΔlacZ #1	0.99
H500 MG1655 #2	G500 ΔlacZ #2	0.88
H500 MG1655 #1	G500 ΔlacZ #1	0.98
H500 MG1655 #2	G500 ΔlacZ #2	0.94

Strain A	Strain B	W
G500 MG1655 #1	P500 ΔlacZ #1	0.98
G500 MG1655 #1	P500 ΔlacZ #2	1.05
G500 MG1655 #2	P500 ΔlacZ #1	1.04
G500 MG1655 #2	P500 ΔlacZ #2	1.00
G500 ΔlacZ #1	P500 MG1655 #1	1.04
G500 ΔlacZ #2	P500 MG1655 #1	1.13
G500 ΔlacZ #1	P500 MG1655 #2	1.02
G500 ΔlacZ #2	P500 MG1655 #2	1.12

Strain A	Strain B	W
B500 ΔlacZ #1	P500 MG1655 #1	0.98
B500 ΔlacZ #2	P500 MG1655 #1	1.04
B500 ΔlacZ #1	P500 MG1655 #2	1.04
B500 ΔlacZ #2	P500 MG1655 #2	1.06
B500 MG1655 #1	P500 ΔlacZ #1	1.08
B500 MG1655 #2	P500 ΔlacZ #1	1.11
B500 MG1655 #1	P500 ΔlacZ #2	1.11
B500 MG1655 #2	P500 ΔlacZ #2	1.11

Strain A	Strain B	W
G500 MG1655 #1	B500 ΔlacZ #1	0.97
G500 MG1655 #1	B500 ΔlacZ #2	0.92
G500 MG1655 #2	B500 ΔlacZ #1	1.06
G500 MG1655 #2	B500 ΔlacZ #2	0.97
G500 ΔlacZ #1	B500 MG1655 #1	0.94
G500 ΔlacZ #1	B500 MG1655 #2	0.92
G500 ΔlacZ #2	B500 MG1655 #1	0.92
G500 ΔlacZ #2	B500 MG1655 #2	0.94

Figure S11. Competition assays on M9+glucose medium. Data in figures represent average of 4 biological and 2 technical replicates. Error bars represent standard error of the mean. Values in corresponding tables show the Darwinian fitness (W) of the individual competition after 48h. (+) indicates no colonies for strain B and thus no calculated fitness value. (A) O500 *vs.* G500; (B) H500 *vs.* G500; (C) G500 *vs.* P500; (D) B500 *vs.* P500; (E) G500 *vs.* B500.



Strain A	Strain B	W
O500 ΔlacZ #1	G500 MG1655 #1	1.26
O500 ΔlacZ #2	G500 MG1655 #1	1.41
O500 ΔlacZ #1	G500 MG1655 #2	1.38
O500 ΔlacZ #2	G500 MG1655 #2	1.36
O500 MG1655 #1	G500 ΔlacZ #1	1.61
O500 MG1655 #2	G500 ΔlacZ #2	1.64
O500 MG1655 #1	G500 ΔlacZ #1	1.39
O500 MG1655 #2	G500 ΔlacZ #2	1.30

Strain A	Strain B	W
B500 ΔlacZ #1	P500 MG1655 #1	1.49
B500 ΔlacZ #2	P500 MG1655 #1	1.14
B500 ΔlacZ #1	P500 MG1655 #2	1.41
B500 ΔlacZ #2	P500 MG1655 #2	1.17
B500 MG1655 #1	P500 ΔlacZ #1	1.28
B500 MG1655 #2	P500 ΔlacZ #2	1.25
B500 MG1655 #1	P500 ΔlacZ #1	1.33
B500 MG1655 #2	P500 ΔlacZ #2	+

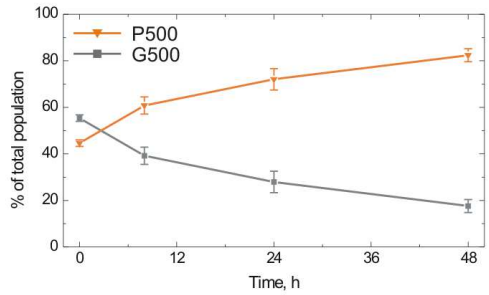
Strain A	Strain B	W
O500 ΔlacZ #1	B500 MG1655 #1	1.01
O500 ΔlacZ #1	B500 MG1655 #2	0.95
O500 ΔlacZ #2	B500 MG1655 #1	0.91
O500 ΔlacZ #2	B500 MG1655 #2	0.92
O500 MG1655 #1	B500 ΔlacZ #1	1.15
O500 MG1655 #1	B500 ΔlacZ #2	1.30
O500 MG1655 #2	B500 ΔlacZ #1	1.10
O500 MG1655 #2	B500 ΔlacZ #2	1.02

Strain A	Strain B	W
B500 ΔlacZ #1	G500 MG1655 #1	1.14
B500 ΔlacZ #1	G500 MG1655 #2	1.49
B500 ΔlacZ #2	G500 MG1655 #1	1.55
B500 ΔlacZ #2	G500 MG1655 #2	1.58
B500 MG1655 #1	G500 ΔlacZ #1	1.41
B500 MG1655 #1	G500 ΔlacZ #2	+
B500 MG1655 #2	G500 ΔlacZ #1	1.47
B500 MG1655 #2	G500 ΔlacZ #2	1.55

Strain A	Strain B	W
H500 ΔlacZ #1	P500 MG1655 #1	1.06
H500 ΔlacZ #2	P500 MG1655 #1	1.01
H500 ΔlacZ #1	P500 MG1655 #2	1.23
H500 ΔlacZ #2	P500 MG1655 #2	1.10
H500 MG1655 #1	P500 ΔlacZ #1	1.08
H500 MG1655 #2	P500 ΔlacZ #2	0.99
H500 MG1655 #1	P500 ΔlacZ #1	0.90
H500 MG1655 #2	P500 ΔlacZ #2	0.87

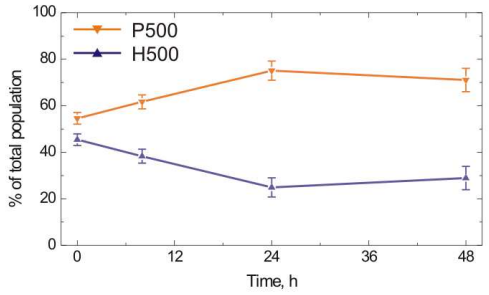
Figure S12. Competition assays under hyper-osmotic stress(0.3M NaCl). Data in figures represent average of 4 biological and 2 technical replicates. Error bars represent standard error of the mean. Values in corresponding tables show the Darwinian fitness (W) of the individual competition after 48h. (+) indicates no colonies for strain B and thus no calculated fitness value. (A) O500 *vs.* G500; (B) B500 *vs.* P500; (C) O500 *vs.* B500; (D) B500 *vs.* G500; (E) H500 *vs.* P500.

A



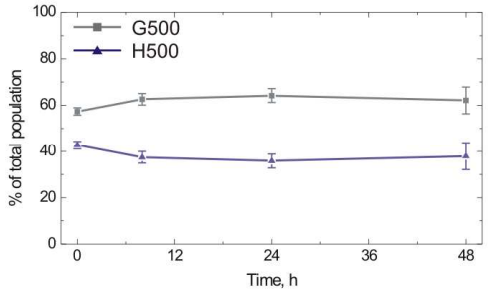
Strain A	Strain B	W
P500 ΔlacZ #1	G500 MG1655 #1	1.23
P500 ΔlacZ #2	G500 MG1655 #1	1.35
P500 ΔlacZ #1	G500 MG1655 #2	1.36
P500 ΔlacZ #2	G500 MG1655 #2	1.39
P500 MG1655 #1	G500 ΔlacZ #1	1.20
P500 MG1655 #2	G500 ΔlacZ #2	1.20
P500 MG1655 #1	G500 ΔlacZ #1	1.17
P500 MG1655 #2	G500 ΔlacZ #2	1.38

B



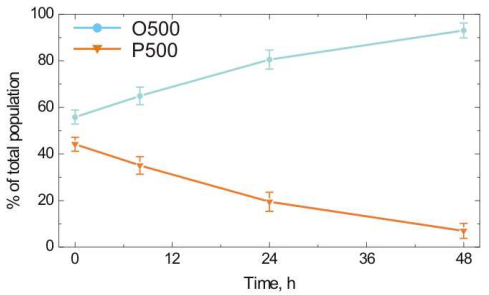
Strain A	Strain B	W
P500 MG1655 #1	H500 ΔlacZ #1	1.12
P500 MG1655 #2	H500 ΔlacZ #2	1.07
P500 MG1655 #1	H500 ΔlacZ #1	0.99
P500 MG1655 #2	H500 ΔlacZ #2	1.04
P500 ΔlacZ #1	H500 MG1655 #1	1.02
P500 ΔlacZ #2	H500 MG1655 #1	1.16
P500 ΔlacZ #1	H500 MG1655 #2	1.13
P500 ΔlacZ #2	H500 MG1655 #2	1.29

C



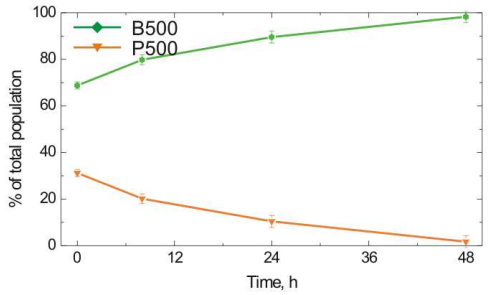
Strain A	Strain B	W
H500 ΔlacZ #1	G500 MG1655 #1	0.85
H500 ΔlacZ #2	G500 MG1655 #1	0.89
H500 ΔlacZ #1	G500 MG1655 #2	1.10
H500 ΔlacZ #2	G500 MG1655 #2	1.11
H500 MG1655 #1	G500 ΔlacZ #1	0.97
H500 MG1655 #2	G500 ΔlacZ #2	0.94
H500 MG1655 #1	G500 ΔlacZ #1	1.00
H500 MG1655 #2	G500 ΔlacZ #2	0.97

D

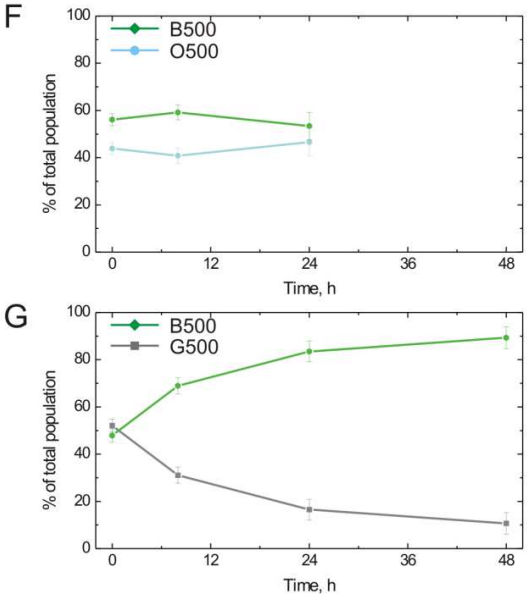


Strain A	Strain B	W
O500 ΔlacZ #1	P500 MG1655 #1	+
O500 ΔlacZ #2	P500 MG1655 #1	1.32
O500 ΔlacZ #1	P500 MG1655 #2	1.33
O500 ΔlacZ #2	P500 MG1655 #2	+
O500 MG1655 #1	P500 ΔlacZ #1	1.28
O500 MG1655 #2	P500 ΔlacZ #2	1.23
O500 MG1655 #1	P500 ΔlacZ #1	1.33
O500 MG1655 #2	P500 ΔlacZ #2	1.12

E



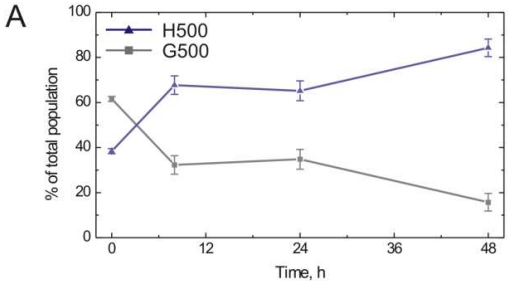
Strain A	Strain B	W
B500 ΔlacZ #1	P500 MG1655 #1	1.25
B500 ΔlacZ #2	P500 MG1655 #1	+
B500 ΔlacZ #1	P500 MG1655 #2	1.34
B500 ΔlacZ #2	P500 MG1655 #2	1.28
B500 MG1655 #1	P500 ΔlacZ #1	+
B500 MG1655 #2	P500 ΔlacZ #2	+
B500 MG1655 #1	P500 ΔlacZ #1	1.25
B500 MG1655 #2	P500 ΔlacZ #2	1.31



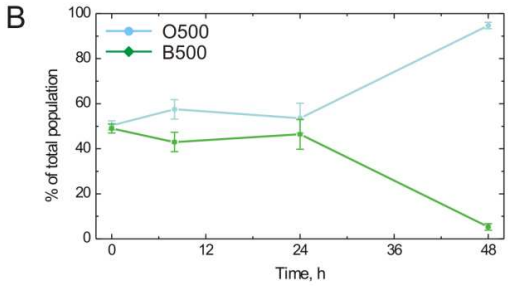
Strain A	Strain B	W
O500 ΔlacZ #1	B500 MG1655 #1	0.91
O500 ΔlacZ #2	B500 MG1655 #1	0.79
O500 ΔlacZ #1	B500 MG1655 #2	1.29
O500 ΔlacZ #2	B500 MG1655 #2	0.94
O500 MG1655 #1	B500 ΔlacZ #1	1.17
O500 MG1655 #2	B500 ΔlacZ #2	1.48
O500 MG1655 #1	B500 ΔlacZ #1	0.86
O500 MG1655 #2	B500 ΔlacZ #2	1.17

Strain A	Strain B	W
B500 ΔlacZ #1	G500 MG1655 #1	1.06
B500 ΔlacZ #2	G500 MG1655 #1	1.54
B500 ΔlacZ #1	G500 MG1655 #2	1.12
B500 ΔlacZ #2	G500 MG1655 #2	1.61
B500 MG1655 #1	G500 ΔlacZ #1	1.49
B500 MG1655 #2	G500 ΔlacZ #2	1.36
B500 MG1655 #1	G500 ΔlacZ #1	1.50
B500 MG1655 #2	G500 ΔlacZ #2	1.41

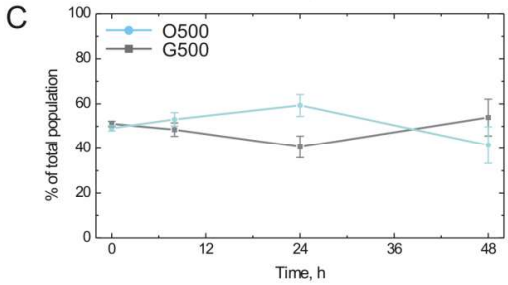
Figure S13. Competition assays under acidic stress (pH 5.5). Data in figures represent average of 4 biological and 2 technical replicates. Error bars represent standard error of the mean. Values in corresponding tables show the Darwinian fitness (W) of the individual competition after 48h. (+) indicates no colonies for strain B and thus no calculated fitness value. (A) P500 *vs.* G500; (B) P500 *vs.* H500; (C) H500 *vs.* G500; (D) O500 *vs.* P500; (E) B500 *vs.* P500; (F) B500 *vs.* O500; (G) B500 *vs.* G500



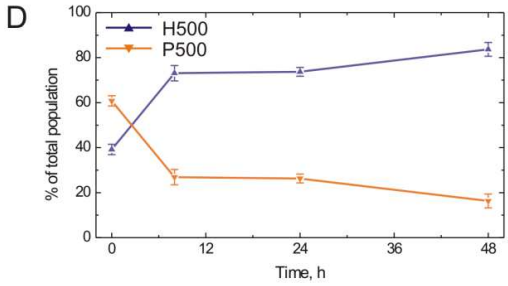
Strain A	Strain B	W
H500 ΔlacZ #1	G500 MG1655 #1	1.07
H500 ΔlacZ #2	G500 MG1655 #1	1.20
H500 ΔlacZ #1	G500 MG1655 #2	1.44
H500 ΔlacZ #2	G500 MG1655 #2	1.47
H500 MG1655 #1	G500 ΔlacZ #1	1.10
H500 MG1655 #2	G500 ΔlacZ #2	1.57
H500 MG1655 #1	G500 ΔlacZ #1	1.52
H500 MG1655 #2	G500 ΔlacZ #2	1.54



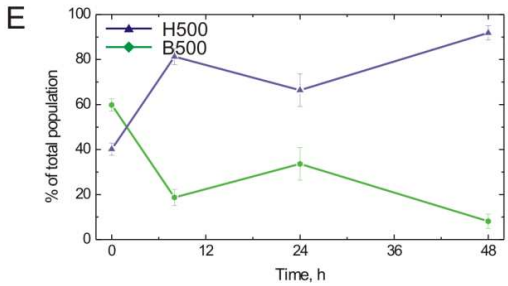
Strain A	Strain B	W
O500 MG1655 #1	B500 ΔlacZ #1	1.55
O500 MG1655 #1	B500 ΔlacZ #2	1.23
O500 MG1655 #2	B500 ΔlacZ #1	1.66
O500 MG1655 #2	B500 ΔlacZ #2	1.59
O500 ΔlacZ #1	B500 MG1655 #1	1.20
O500 ΔlacZ #1	B500 MG1655 #2	1.34
O500 ΔlacZ #2	B500 MG1655 #1	*nd*
O500 ΔlacZ #2	B500 MG1655 #2	*nd*



Strain A	Strain B	W
G500 MG1655 #1	O500 ΔlacZ #1	1.01
G500 MG1655 #1	O500 ΔlacZ #2	1.34
G500 MG1655 #2	O500 ΔlacZ #1	0.92
G500 MG1655 #2	O500 ΔlacZ #2	+
G500 ΔlacZ #1	O500 MG1655 #1	0.92
G500 ΔlacZ #1	O500 MG1655 #2	0.83
G500 ΔlacZ #2	O500 MG1655 #1	1.05
G500 ΔlacZ #2	O500 MG1655 #2	1.07

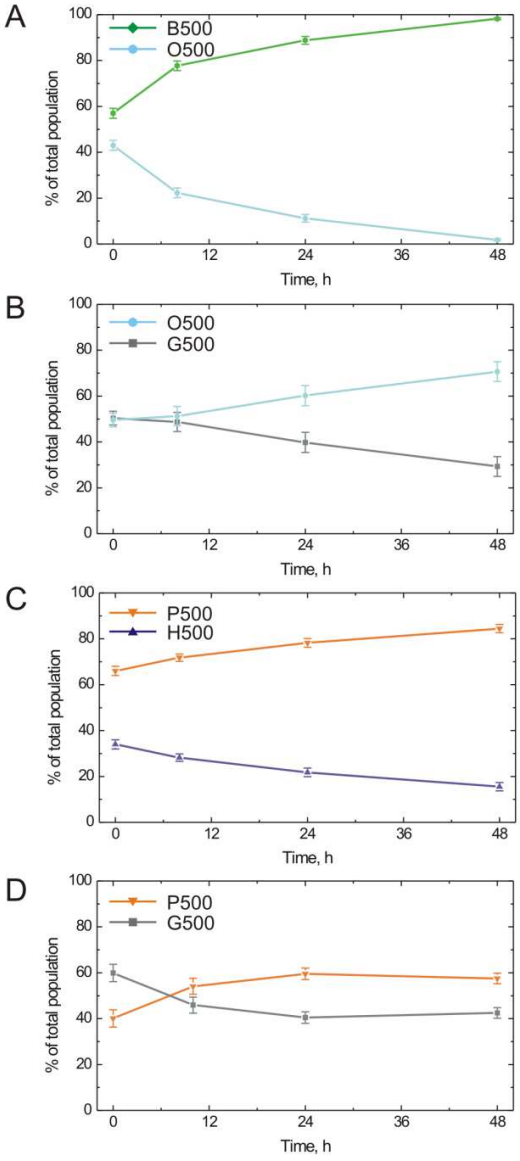


H500 ΔlacZ #1	P500 MG1655 #1	1.07
H500 ΔlacZ #2	P500 MG1655 #1	1.10
H500 ΔlacZ #1	P500 MG1655 #2	1.20
H500 ΔlacZ #2	P500 MG1655 #2	1.14
H500 MG1655 #1	P500 ΔlacZ #1	1.29
H500 MG1655 #2	P500 ΔlacZ #1	1.56
H500 MG1655 #1	P500 ΔlacZ #2	1.24
H500 MG1655 #2	P500 ΔlacZ #2	1.50
H500 ΔlacZ #1	P500 MG1655 #1	1.07



Strain A	Strain B	W
H500 ΔlacZ #1	B500 MG1655 #1	1.19
H500 ΔlacZ #2	B500 MG1655 #1	1.27
H500 ΔlacZ #1	B500 MG1655 #2	1.30
H500 ΔlacZ #2	B500 MG1655 #2	1.22
H500 MG1655 #1	B500 ΔlacZ #1	1.61
H500 MG1655 #2	B500 ΔlacZ #1	1.58
H500 MG1655 #1	B500 ΔlacZ #2	1.43
H500 MG1655 #2	B500 ΔlacZ #2	+

Figure S14. Competition assays under oxidative stress (100μM H_2O_2). Data in figures represent average of 4 biological and 2 technical replicates. Error bars represent standard error of the mean. Values in corresponding tables show the observed Darwinian fitness (W) of the individual competition after 48h. *nd* – no data available (+) indicates no colonies for strain B and thus no calculated fitness value. (A) G500 *vs.* H500; (B) O500 *vs.* B500; (C) G500 *vs.* O500; (D) H500 *vs.* P500; (E) H500 *vs.* B500.



Strain A	Strain B	W
B500 MG1655 #1	O500 ΔlacZ #1	1.37
B500 MG1655 #2	O500 ΔlacZ #2	1.30
B500 MG1655 #1	O500 ΔlacZ #1	1.48
B500 MG1655 #2	O500 ΔlacZ #2	1.55
B500 ΔlacZ #1	O500 MG1655 #1	1.30
B500 ΔlacZ #2	O500 MG1655 #1	1.54
B500 ΔlacZ #1	O500 MG1655 #2	1.51
B500 ΔlacZ #2	O500 MG1655 #2	1.48

Strain A	Strain B	W
O500 ΔlacZ #1	G500 MG1655 #1	1.19
O500 ΔlacZ #2	G500 MG1655 #1	1.17
O500 ΔlacZ #1	G500 MG1655 #2	1.11
O500 ΔlacZ #2	G500 MG1655 #2	1.10
O500 MG1655 #1	G500 ΔlacZ #1	1.06
O500 MG1655 #2	G500 ΔlacZ #2	1.08
O500 MG1655 #1	G500 ΔlacZ #1	1.06
O500 MG1655 #2	G500 ΔlacZ #2	1.07

Strain A	Strain B	W
P500 ΔlacZ #1	H500 MG1655 #1	1.06
P500 ΔlacZ #2	H500 MG1655 #1	1.08
P500 ΔlacZ #1	H500 MG1655 #2	1.01
P500 ΔlacZ #2	H500 MG1655 #2	1.08
P500 MG1655 #1	H500 ΔlacZ #1	1.07
P500 MG1655 #2	H500 ΔlacZ #2	1.09
P500 MG1655 #1	H500 ΔlacZ #1	1.13
P500 MG1655 #2	H500 ΔlacZ #2	1.12

Strain A	Strain B	W
P500 ΔlacZ #1	G500 MG1655 #1	1.19
P500 ΔlacZ #2	G500 MG1655 #1	1.17
P500 ΔlacZ #1	G500 MG1655 #2	1.11
P500 ΔlacZ #2	G500 MG1655 #2	1.10
P500 MG1655 #1	G500 ΔlacZ #1	1.06
P500 MG1655 #2	G500 ΔlacZ #2	1.08
P500 MG1655 #1	G500 ΔlacZ #1	1.07
P500 MG1655 #2	G500 ΔlacZ #2	1.07

Figure S15. Competition assays under *n*-butanol stress (0.6% *n*-butanol). Data in figures represent average of 4 biological and 2 technical replicates. Error bars represent standard error of the mean. Values in corresponding tables show the Darwinian fitness (W) of the individual competition after 48h. (+) indicates no colonies for strain B and thus no calculated fitness value. (A) B500 *vs.* O500; (B) O500 *vs.* G500; (C) H500 *vs.* P500; (D) P500 *vs.* G500.

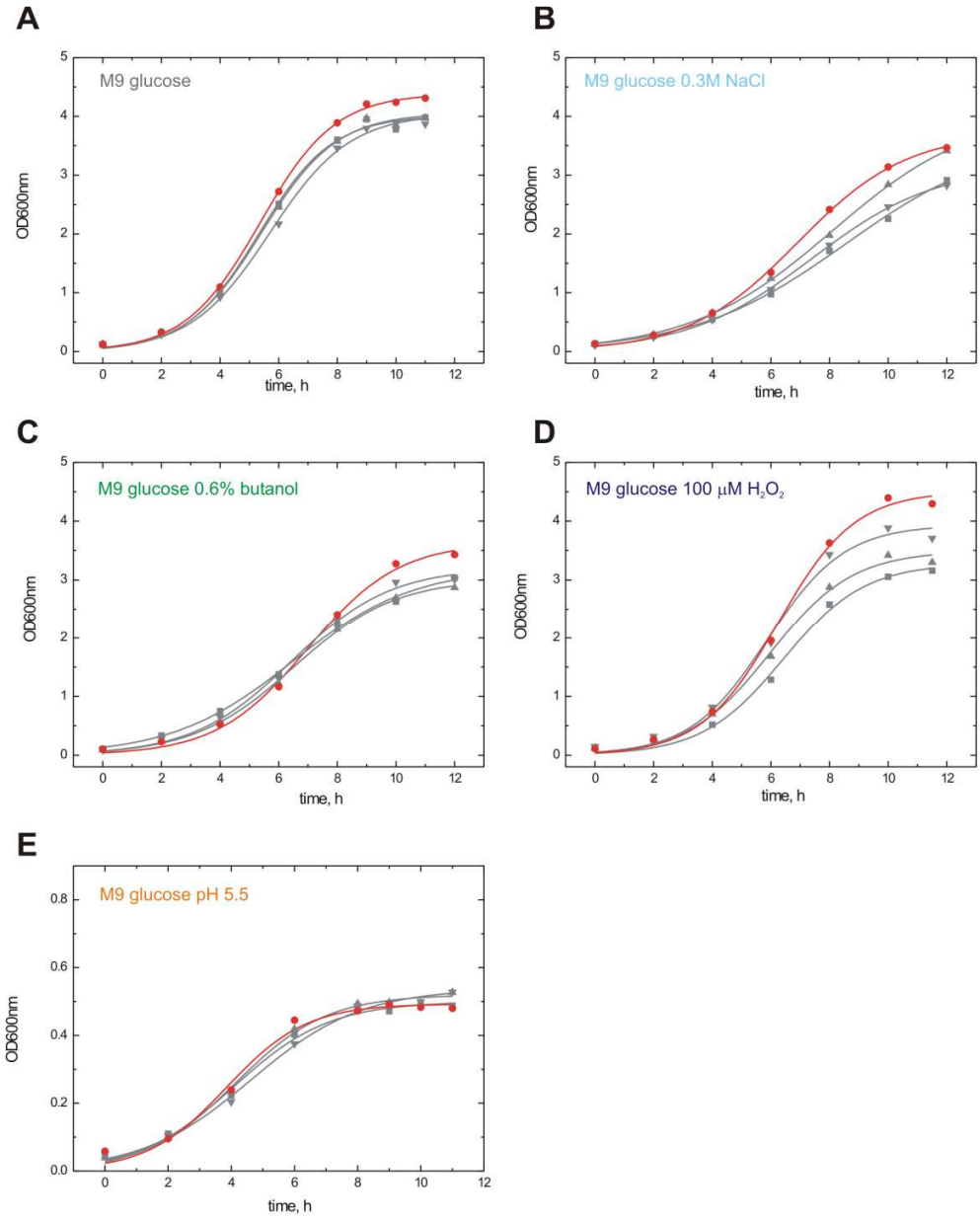


Figure S16. Growth curves of 4 individual clones on the biological replicates selected for sequencing. Red curves represent the growth curve of the clone selected for genomic sequencing. (A) G500 under no stress; (B) O500 under hyper-osmotic stress (0.3M NaCl); (C) B500 under *n*-butanol stress (0.6% *n*-butanol); (D) H500 under oxidative stress (100mM H_2O_2); (E) P500 under acidic stress (pH 5.5). Experimental data (2 technical replicates per growth curve) were fitted by sigmoidal logistic curve fitting.

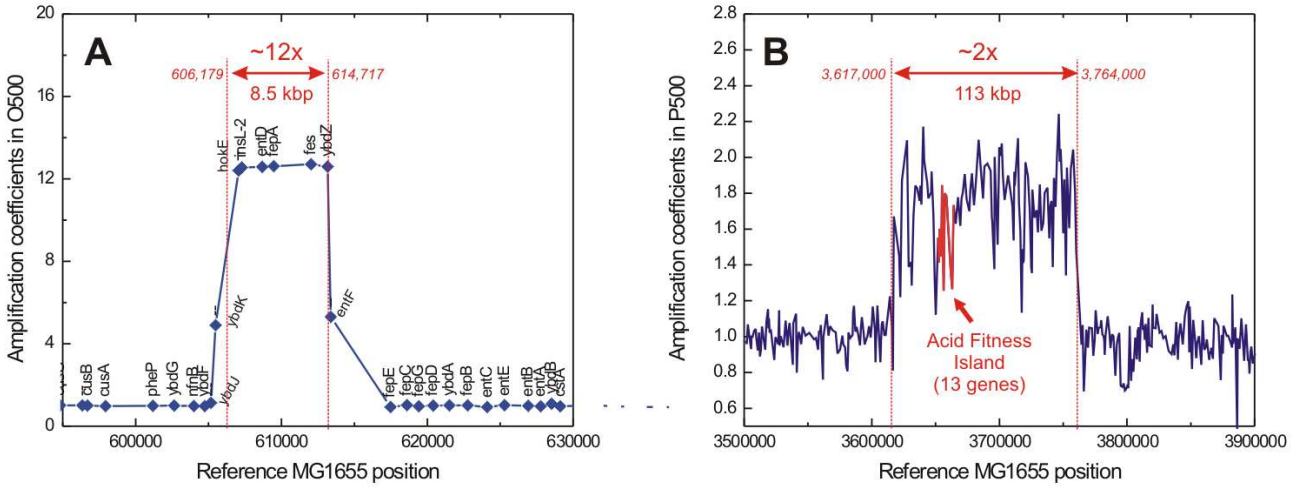


Figure S17. Average coverage increase observed by resequencing for amplified genes. (A) O500 and (B) P500 strain**.** Amplification coefficient for each gene is calculated as a ratio between the numbers of read-counts per gene in a strain with amplification to the average read-count per gene in all other strains with no amplification.

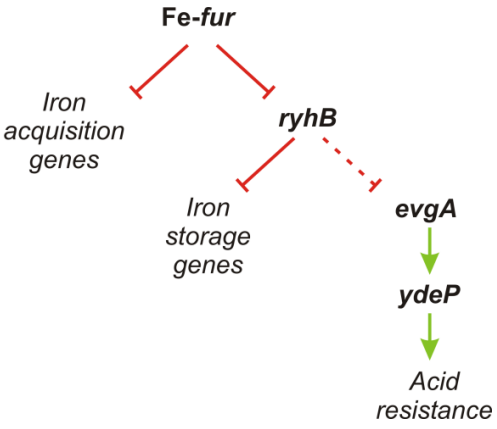


Figure S18. **Putative iron-related acid resistance response pathway in *E. coli*.** Proposed interaction between iron and acid stress response pathways in *E. coli* based on the known *E. coli* regulatory network and the *Shigella flexneri* study (Oglesby et al, 2005). Dashed inhibitory link between *ryhB* and *evgA* is not in the current *E. coli* pathways databases.

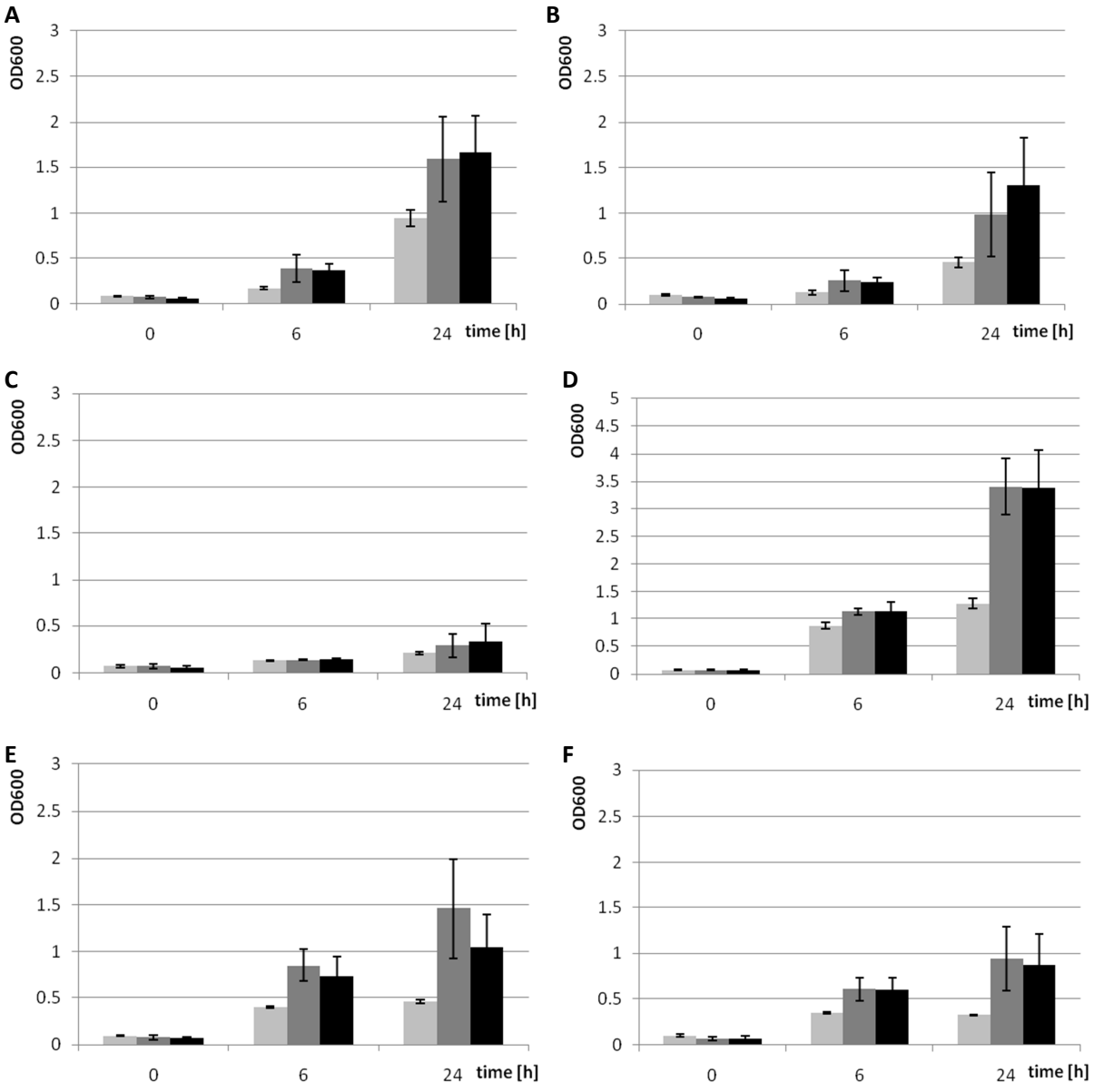


Figure S19. ***n*-Butanol stress tolerance in M9 and LB media.** Growth test of ancestral strains (light grey), B500 strains (dark grey) and B1000 strains (black) on increased n-butanol concentrations in the growth medium. (A-C) M9 medium containing 0.8, 1.0 and 1.2% n-butanol. (D-F) LB medium containing 0.8, 1.0 and 1.2% butanol.

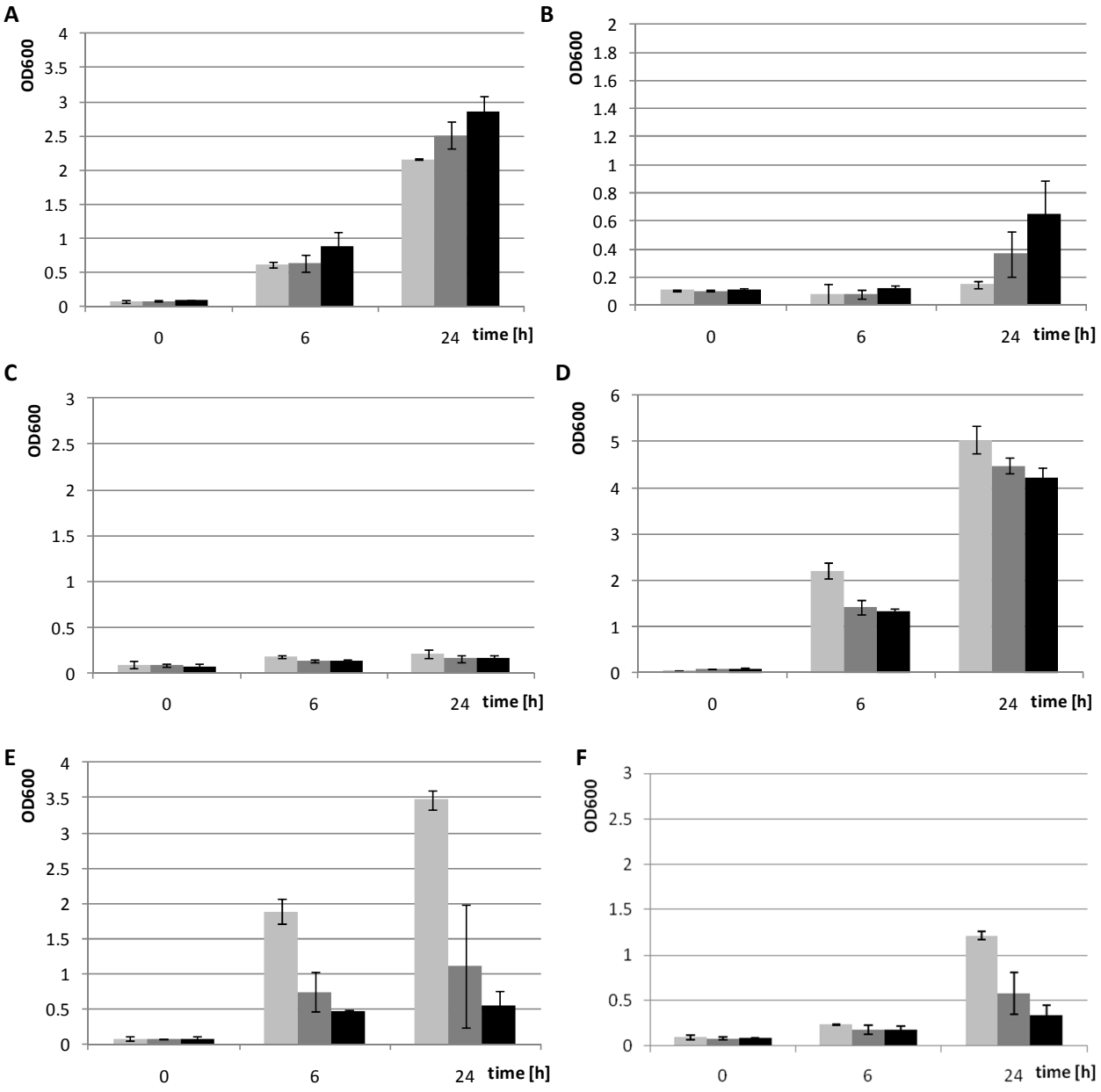


Figure S20. **Osmotic stress tolerance in M9 and LB media.** Growth test of ancestral strains (light grey), O500 strains (dark grey) and O1000 strains (black) on increased n-butanol concentrations in the growth medium. (A-C) M9 medium containing 2.25, 5% and 6% NaCl. (D-F) LB medium containing 4, 6 and 8% NaCl.

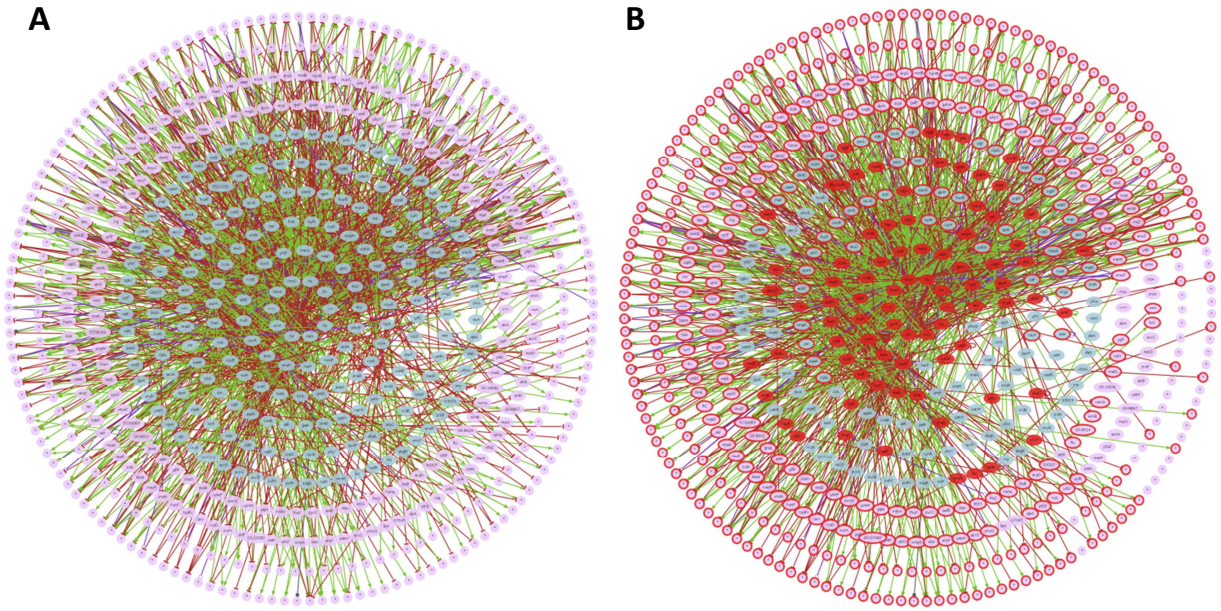


Figure S21. Reconstructed regulatory network of *E. coli* with DE genes. (A) An overview of the reconstructed gene regulatory network of *E. coli* MG 1655 strain. Blue and pink nodes are regulators and terminal genes (leaves), respectively. Higher level regulators are closer to the center. Regulated genes from the same transcription unit and identical regulation are grouped together. (B) Gene regulatory sub-network connected to the differentially expressed genes (DE regulators are shown as solid red nodes) in stress evolved strains; nodes which are direct or indirect regulators of DE genes or being regulated by these regulators are encircled in red.

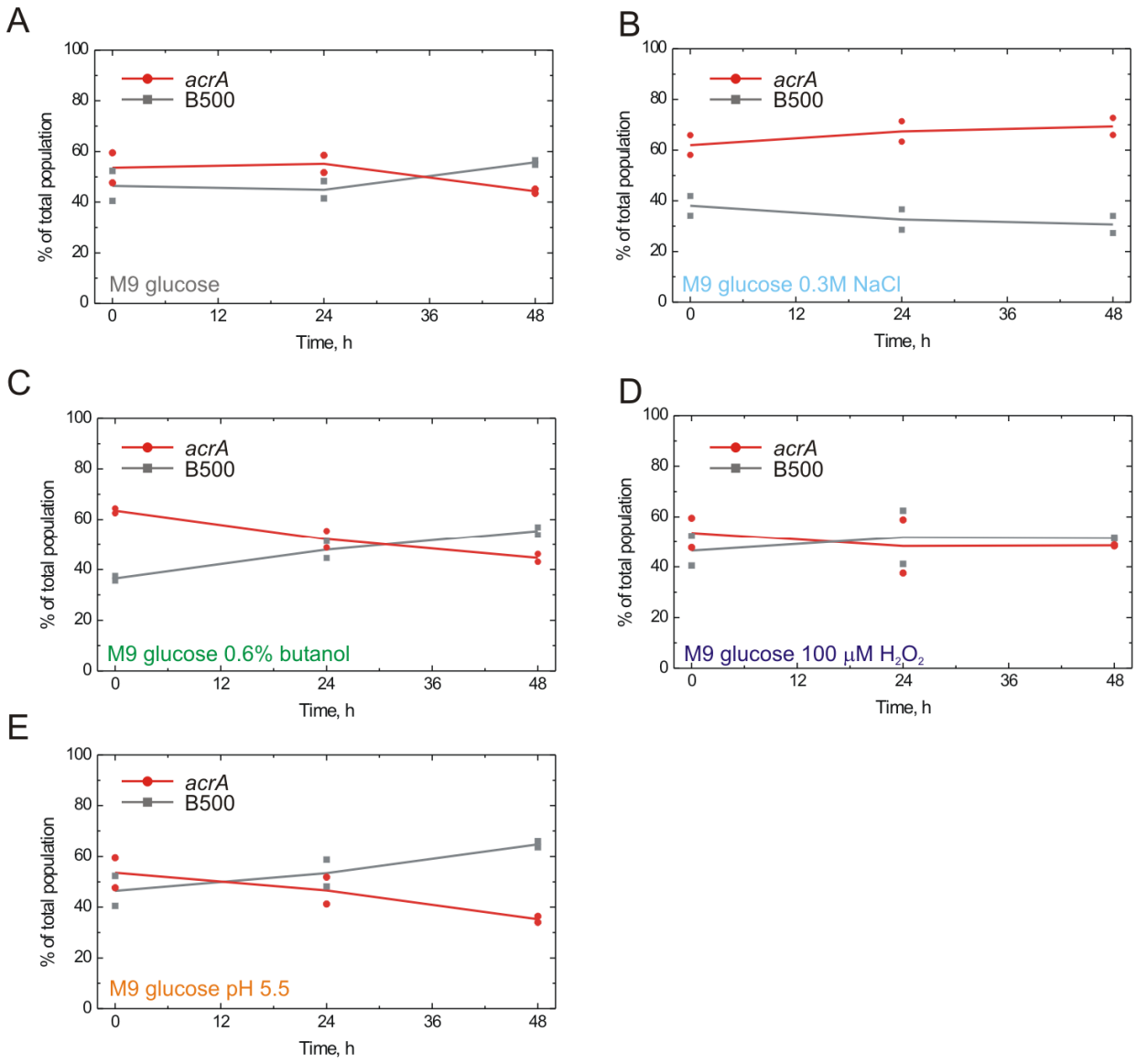


Figure S22. Competition assays for B500 *acrA* repair mutant. Red lines: B500 *acrA* repair mutant; gray lines: B500. (A) No stress; (B) hyper-osmotic stress (0.3M NaCl); (C) *n*-butanol stress (0.6% *n*-butanol); (D) oxidative stress (100mM H_2O_2); (E) acidic stress (pH 5.5). The medium is always M9 salt with glucose as a sole carbon source. Lines represent averages of 2 technical replicates (shown with dots).

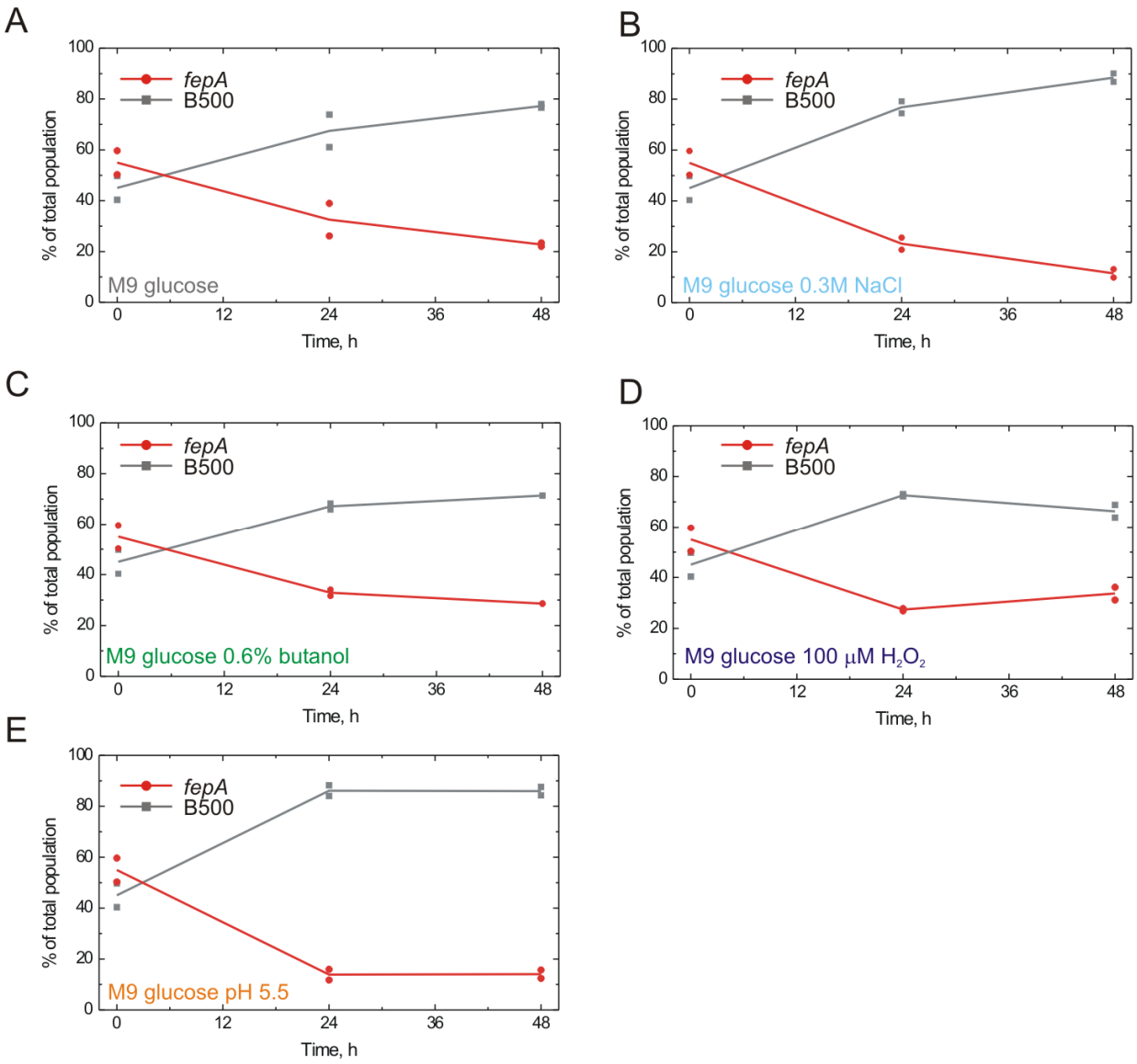


Figure S23. Competition assays for B500 *fepA* repair mutant. Red lines: B500 *fepA* repair mutant; gray lines: B500. (A) No stress; (B) hyper-osmotic stress (0.3M NaCl); (C) *n*-butanol stress (0.6% *n*-butanol); (D) oxidative stress (100mM H_2O_2); (E) acidic stress (pH 5.5). The medium is always M9 salt with glucose as a sole carbon source. Lines represent averages of 2 technical replicates (shown with dots).

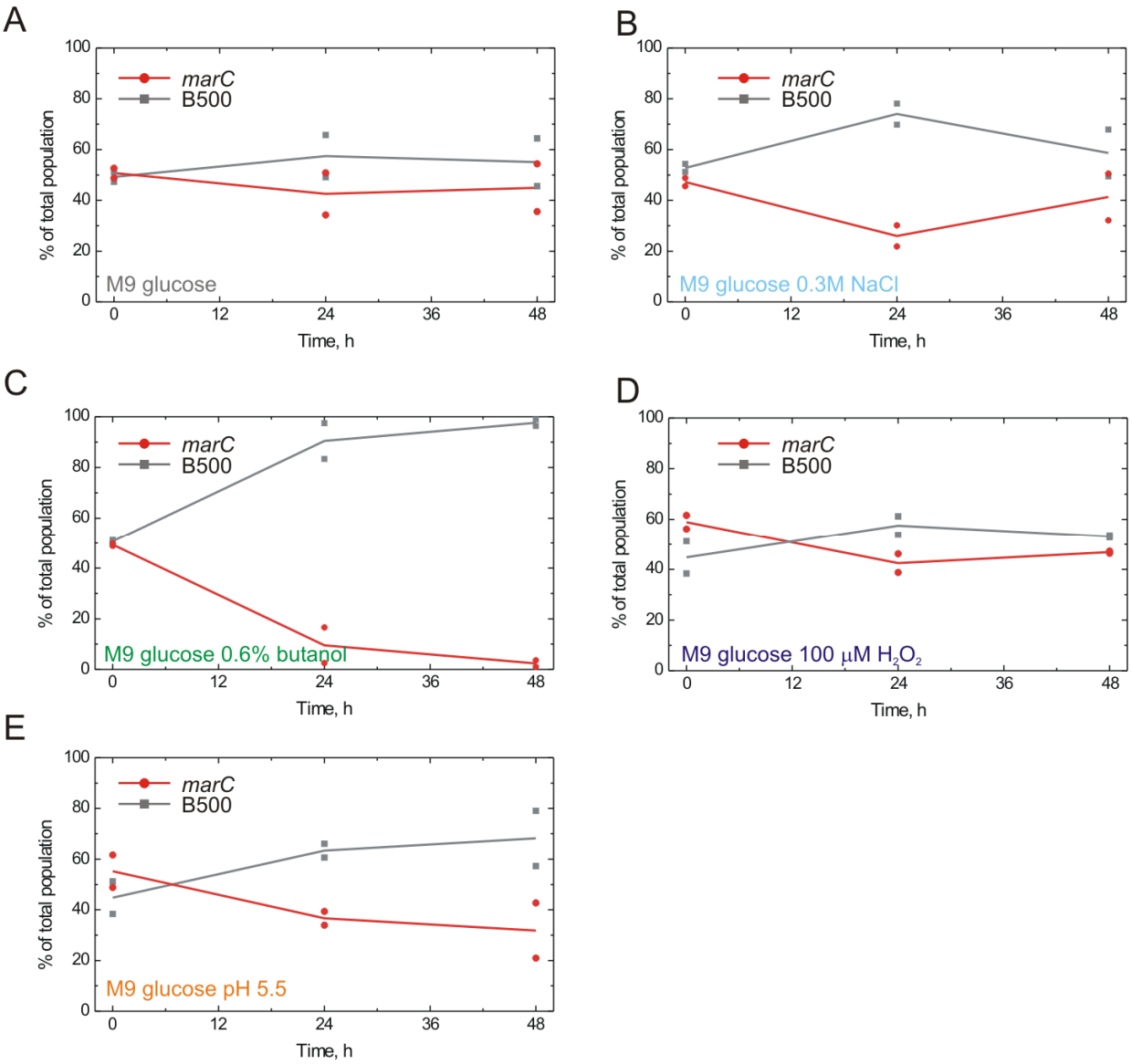


Figure S24. Competition assays for B500 *marC* repair mutant. Red lines: B500 *marC* repair mutant; gray lines: B500. (A) No stress; (B) hyper-osmotic stress (0.3M NaCl); (C) *n*-butanol stress (0.6% *n*-butanol); (D) oxidative stress (100mM H_2O_2); (E) acidic stress (pH 5.5). The medium is always M9 salt with glucose as a sole carbon source. Lines represent averages of 2 technical replicates (shown with dots).

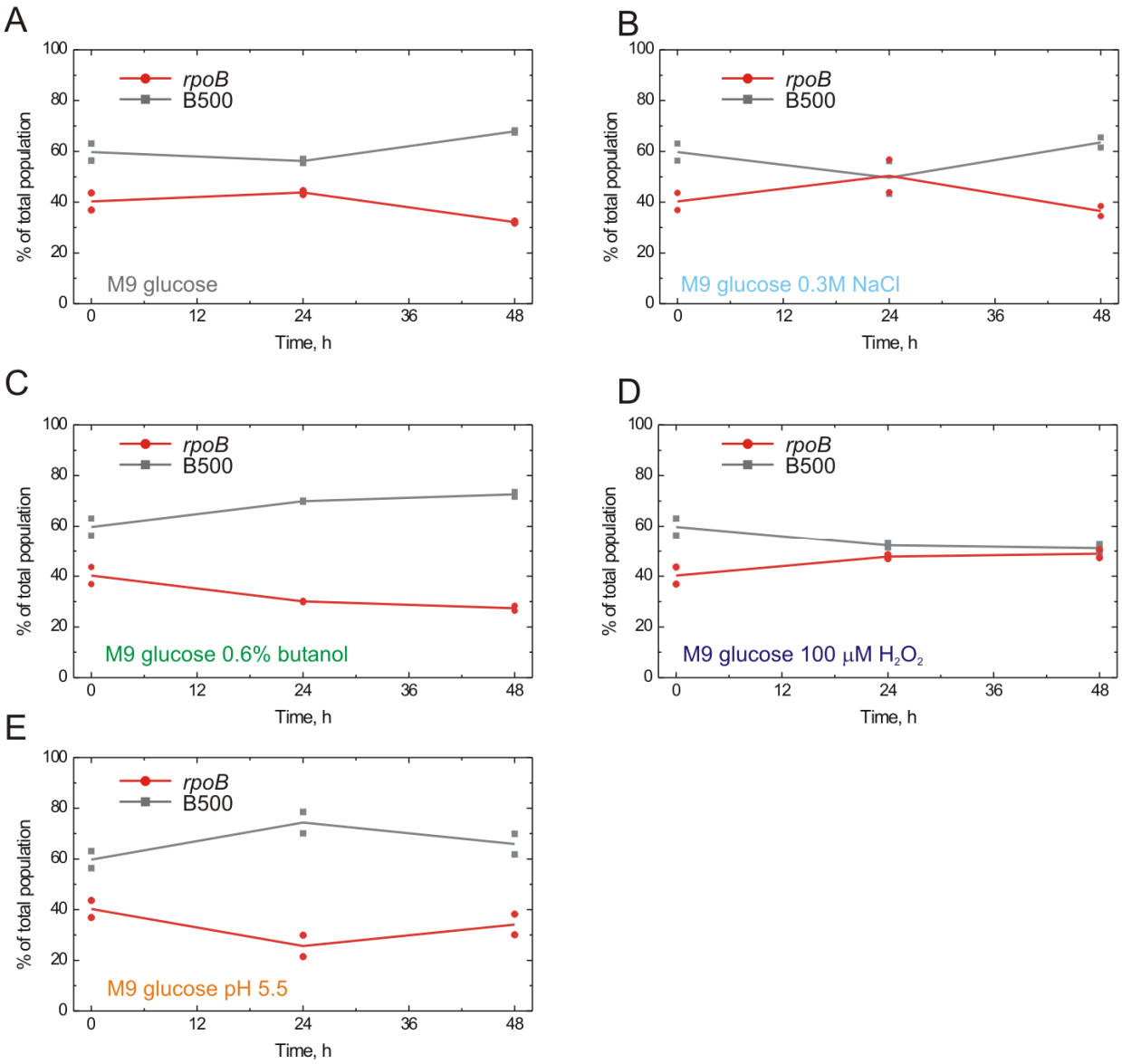


Figure S25. Competition assays for B500 *rpoB* repair mutant. Red lines: B500 *rpoB* repair mutant; gray lines: B500. (A) No stress; (B) hyper-osmotic stress (0.3M NaCl); (C) *n*-butanol stress (0.6% *n*-butanol); (D) oxidative stress (100mM H_2O_2); (E) acidic stress (pH 5.5). The medium is always M9 salt with glucose as a sole carbon source. Lines represent averages of 2 technical replicates (shown with dots).

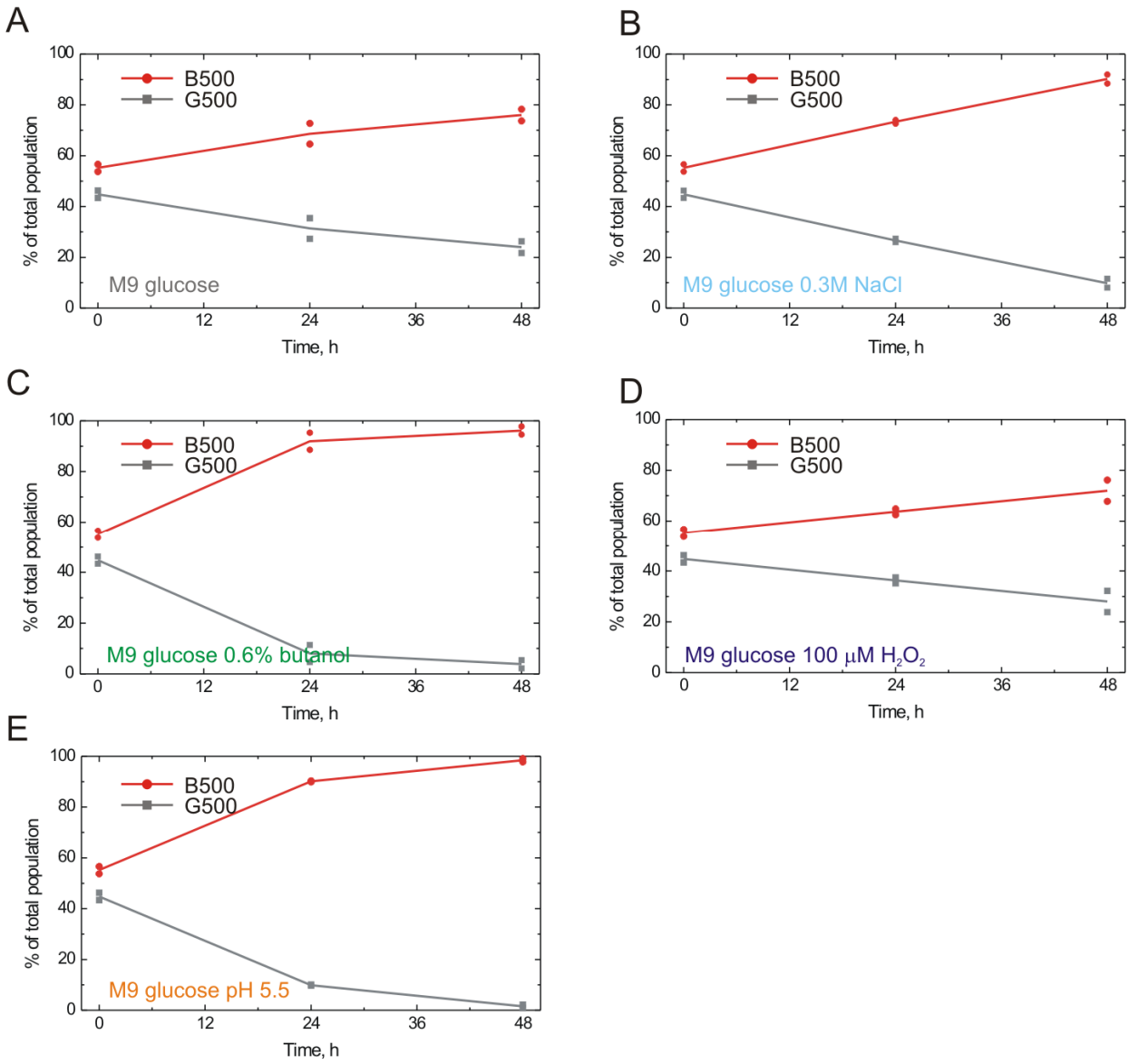


Figure S26. Competition assays for B500 (red) vs. G500 (gray) clones. (A) No stress; (B) hyper-osmotic stress (0.3M NaCl); (C) *n*-butanol stress (0.6% *n*-butanol); (D) oxidative stress (100mM H_2O_2); (E) acidic stress (pH 5.5). The medium is always M9 salt with glucose as a sole carbon source. Lines represent averages of 2 technical replicates (shown with dots).

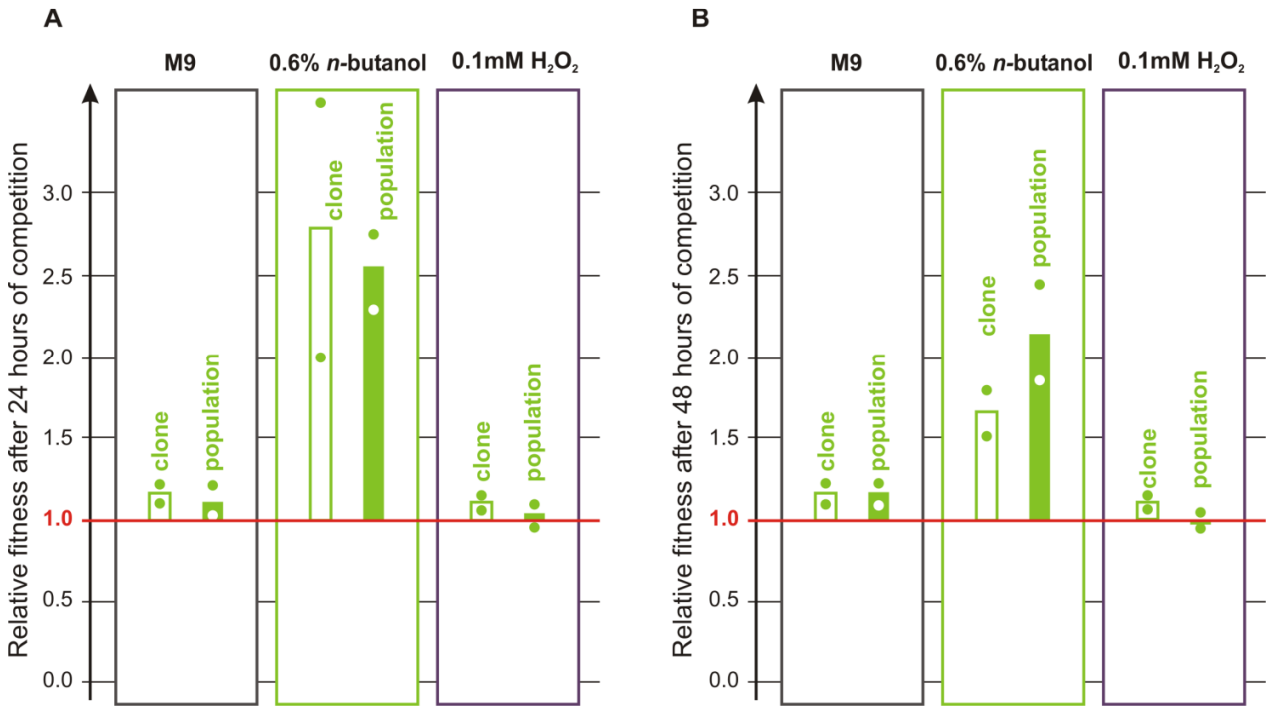


Figure S27. Relative fitness of evolved B500 clones (empty bars) and evolved B500 populations (filled bars) relative to the reference G500 population after (A) 24 hours and (B) 48 hours of growth. Competition assays where performed under the following environmental conditions: (a) no stress, (b) *n*-butanol stress (0.6% *n*-butanol), and (c) oxidative stress (100mM H_2O_2). The medium is always M9 salt with glucose as a sole carbon source. Bars represent averages of 2 technical replicates (shown with dots).

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