Yeast Adapts to a Changing Stressful Environment by Evolving Cross-Protection and Anticipatory Gene Regulation

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Abstract

Organisms can protect themselves against future environmental change. An example is cross-protection, where physiological adaptation against a present environmental stressor can protect an organism against a future stressor. Another is anticipation, where an organism uses information about its present environment to trigger gene expression and other physiological changes adaptive in future environments. "Predictive" abilities like this exist in organisms that have been exposed to periodic changes in environments. It is unknown how readily they can evolve. To answer this question, we carried out laboratory evolution experiments in the yeast *Saccharomyces cerevisiae*. Specifically, we exposed three replicate populations of yeast to environments that varied cyclically between two stressors, salt stress and oxidative stress, every 10 generations, for a total of 300 generations. We evolved six replicate control populations in only one of these stressors for the same amount of time. We analyzed fitness changes and genome-scale expression changes in all these evolved populations. Our populations evolved asymmetric cross protection, where oxidative stress protects against salt stress but not vice versa. Gene expression data also suggest the evolution of anticipation and basal gene expression changes that occur uniquely in cyclic environments. Our study shows that highly complex physiological states that are adaptive in future environments can evolve on very short evolutionary time scales.

Key words: experimental evolution, yeast, stress adaptation, transcriptomics, cross-protection, anticipation.

Introduction

Organisms are continually challenged by changing environments. They thus have evolved physiological adaptations to cope with such change. These mechanisms include genome-scale gene expression changes on short, physiological time scales. Such changes have been most thoroughly studied in stressful environments, such as environments characterized by osmotic stress, oxidative stress, or temperature stress (Jamieson 1998; Gasch et al. 2000; Posas et al. 2000; Rep et al. 2000; Causton et al. 2001; Petersohn et al. 2001; Yale and Bohnert 2001; Zheng et al. 2001; Weber and Jung 2002; Toledano et al. 2003; Weber et al. 2005; Gasch 2007; Chen et al. 2008; Jozefczuk et al. 2010). Some genes respond to multiple stressors in a "general" or "common" environmental stress response, whereas others respond to specific stressors (Gasch et al. 2000; Causton et al. 2001; Petersohn et al. 2001; Higgins et al. 2002; Warringer et al. 2003; Chinnusamy et al. 2004; Phadtare and Inouye 2004; Weber et al. 2005; Yoshikawa et al. 2009; Rutherford et al. 2010).

Some environmental stressors may be extremely rare, whereas others may occur more frequently, and yet others may even recur on a regular basis, such as fluctuation in temperature and nutrient level in circadian cycles (Wijnen and Young 2006; van der Linden et al. 2010), or changes in soil properties over longer periods of time (Fierer et al. 2003; Bapiri et al. 2010; DeAngelis et al. 2010). Several physiological

mechanisms can help organisms prepare for recurring stressors. First, rare and stochastic recurrences may require stochastic switching of gene expression (Thattai and van Oudenaarden 2004; Kussell and Leibler 2005; Blake et al. 2006; Beaumont et al. 2009; Salathé et al. 2009; Gaál et al. 2010; Rainey et al. 2011). This is a form of bet-hedging (Montgomery 1974; Philippi and Seger 1989; de Jong et al. 2011) where different, otherwise identical individuals of a population express different genes, which may protect their carriers against specific stressors (Attfield et al. 2001; Booth 2002; Meyers and Bull 2002; Sumner and Avery 2002). Although most of the population would be vulnerable to the stressor, a small part of it would be protected.

A second relevant mechanism is evolved cross-protection. In cross-protection, one environmental stressor protects cells against a second stressor (Völker et al. 1992; Cullum et al. 2001; Greenacre and Brocklehurst 2006; Berry and Gasch 2008; Berry et al. 2011). For example, exposure of yeast cells to salt stress can improve their fitness in oxidative stress (Berry and Gasch 2008; Berry et al. 2011). Cross-protection is by no means universal among stressors. That is, not every stressor cross-protects against other stressors (Völker et al. 1992; Berry and Gasch 2008; Berry et al. 2011). We know little about whether and how cross-protection can change in evolution.

The third mechanism is anticipation. Here, a present environment serves as a signal for future environmental change

(Tagkopoulos et al. 2008; Wolf et al. 2008; Mitchell et al. 2009). Cells use this signal to preadapt to the future change. An example involves the environmental changes that *Escherichia coli* typically experiences as it is ingested by a mammal. Specifically, ambient temperature increases rapidly immediately after ingestion, and then ambient oxygen levels decrease as cells enter the gastrointestinal tract. *Escherichia coli* cells exposed to high temperatures express genes that may be adaptive for the subsequent drop in oxygen levels (Tagkopoulos et al. 2008). Another example is exposure to lactose, which helps prepare *E. coli* cells for exposure to maltose. This anticipation reflects the temporal order in which these sugars appear in the mammalian digestive tract (Mitchell et al. 2009). Similarly, exposure of yeast cells to heat stress helps them prepare for oxidative stress. The two stressors may follow one another during the wine production process for which yeasts are indispensable (Mitchell et al. 2009). Analogous predictive mechanisms are present in *Candida albicans* and *Vibrio cholerae* (Schild et al. 2007; Rodaki et al. 2009). Theoretical work has studied the costs and benefits of such adaptive predictions (Mitchell and Pilpel 2011).

In contrast to well-studied "physiological" adaptations to stressful environments, the process of "evolutionary" adaptations to such environments is less well studied (Völker et al. 1992; Jamieson 1998; Ferea et al. 1999; Gasch et al. 2000; Posas et al. 2000; Rep et al. 2000; Causton et al. 2001; Petersohn et al. 2001; Yale and Bohnert 2001; Zheng et al. 2001; Weber and Jung 2002; Toledano et al. 2003; Weber et al. 2005; Greenacre and Brocklehurst 2006; Gasch 2007; Chen et al. 2008; Jozefczuk et al. 2010; Cullum et al. 2001; Cooper et al. 2003; Alcántara-Díaz et al. 2004; Fong et al. 2005; Pelosi et al. 2006; Bennett and Lenski 2007; Hughes et al. 2007a, 2007b; Jasmin and Kassen 2007; Cooper and Lenski 2010; Rudolph et al. 2010; Samani and Bell 2010; Dhar et al. 2011; Goldman and Travisano 2011). We here use laboratory evolution to study evolutionary adaptation of yeast cells to "changing" stressful environments, an especially poorly studied subject. Among the small amount of relevant work is one study that asked how *E. coli* cells adapt evolutionarily to fluctuating acidity. It showed that environmental generalists emerge which are better adapted to varying acidity than their ancestors (Hughes et al. 2007b) but left the mechanisms behind their adaptation open. Another study showed that anticipation can readily be reduced on short evolutionary time scales (Tagkopoulos et al. 2008) but left open the question whether anticipation can arise just as easily.

In our experiment, we chose two cyclically varying environments that are characterized by different stressors. Specifically, the stressors we used are salt stress caused by sodium chloride (NaCl) and oxidative stress caused by hydrogen peroxide. NaCl causes hyperosmotic stress to yeast cells. In addition, it imposes hyperionic stress on cells due to the presence of high concentrations of Na^+ and Cl^- ions and necessitates ion detoxification mechanisms (Apse et al. 1999; Maathuis and Amtmann 1999; Serrano et al. 1999; Hohmann 2002). Hydrogen peroxide generates reactive oxygen species that are responsible for oxidative damage

through the oxidation of metabolites, enzymes, and DNA, thus inhibiting metabolism and growth (Jamieson 1998; Toledano et al. 2003).

We evolved three yeast populations in parallel, in an environment characterized by cyclically varying stressors. In the first part of each environmental cycle, we exposed the cells to salt stress (0.5 M NaCl) for 10 generations. In the second part, we exposed them to oxidative stress (1 mM hydrogen peroxide) for another 10 generations. We alternated between these environments for a total of 300 generations, that is, for 15 cycles and called the yeast populations thus evolved SO populations (fig. 1a). In control experiments, we evolved three yeast populations under continuous salt stress for 300 generations (S populations), and yet another three populations under continuous oxidative stress for 300 generations (O populations) (fig. 1a).

To study the evolutionary adaptation of all nine populations to our stressors, we carried out competition assays to measure the fitness of evolved populations relative to the ancestral strain, using a yeast strain labeled with green fluorescent protein (GFP; see Materials and Methods). To understand functional genomic changes that may be associated with these fitness changes, we used gene expression microarrays to study transcriptome-wide gene expression changes in the evolved and ancestral populations.

With these experiments, we asked several questions. Does evolutionary adaptation to salt stress and oxidative stress reflect a general stress response or a response specific to a given stressor? Are there trade-offs between fitness in the two stressors? And most importantly, do abilities such as cross-protection or anticipation evolve? We found that within a mere 300 generations, our populations adapted evolutionarily to the fluctuating stressors. They evolved changes in fitness and gene expression that suggest several, nonexclusive mechanisms to cope with cyclical change, including cross-protection, anticipation, and a change in basal gene expression levels.

Results

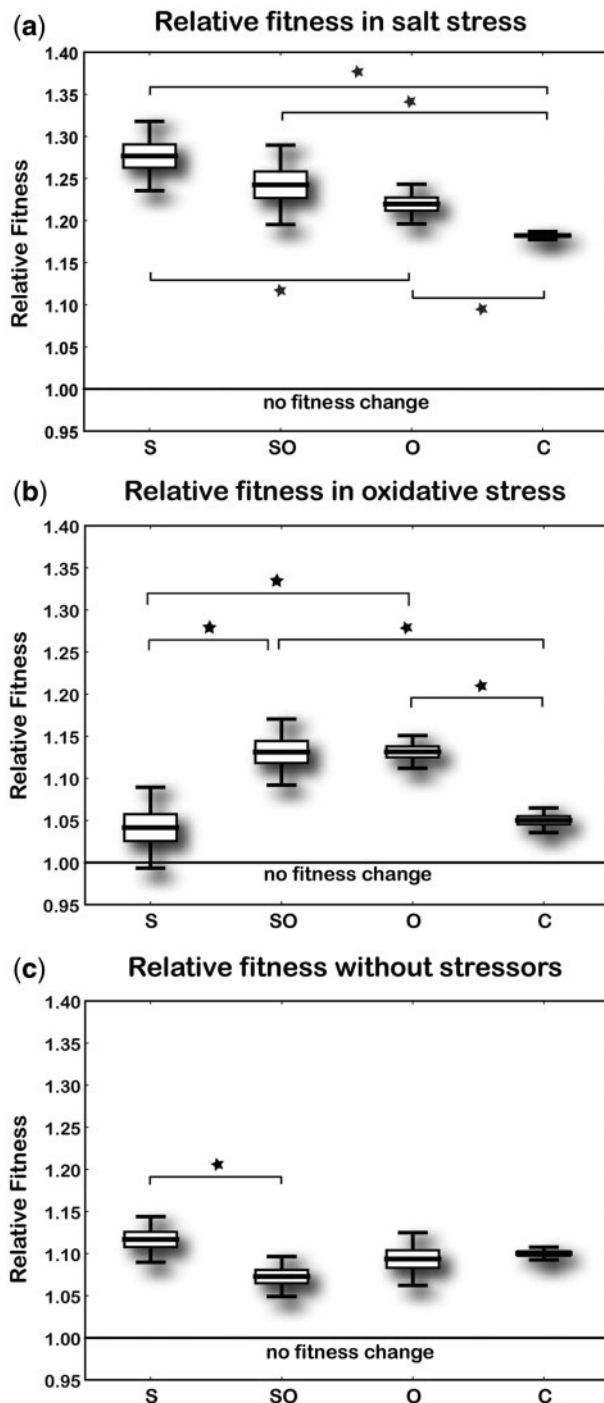### Populations Adapt Evolutionarily in a Partially Stressor-Specific Manner

Our study populations evolved increased fitness in both salt stress and oxidative stress (fig. 2). Specifically, when exposed to salt stress, the salt-evolved (S-) populations increased their fitness relative to the ancestor ($w = 1.28 \pm 0.04$; fig. 2a). Their fitness also increased when measured in an environment without salt stress but to a significantly lesser extent (Mann–Whitney U test, $P = 4 \times 10^{-5}$; fig. 2a and c; see also Dhar et al. 2011). The peroxide-evolved (O-) populations also increased their fitness in oxidative stress relative to the ancestor ($w = 1.13 \pm 0.02$; $P = 0.004$; one sample Wilcoxon signed rank test). Their fitness increased in the absence of oxidative stress as well but to a significantly lesser extent (Mann–Whitney U test, $P = 0.004$; fig. 2a and c). And the same holds for the SO populations, which cycle between two environments (fig. 2; Mann–Whitney U tests, $P = 0.004$ in salt stress and in oxidative stress).
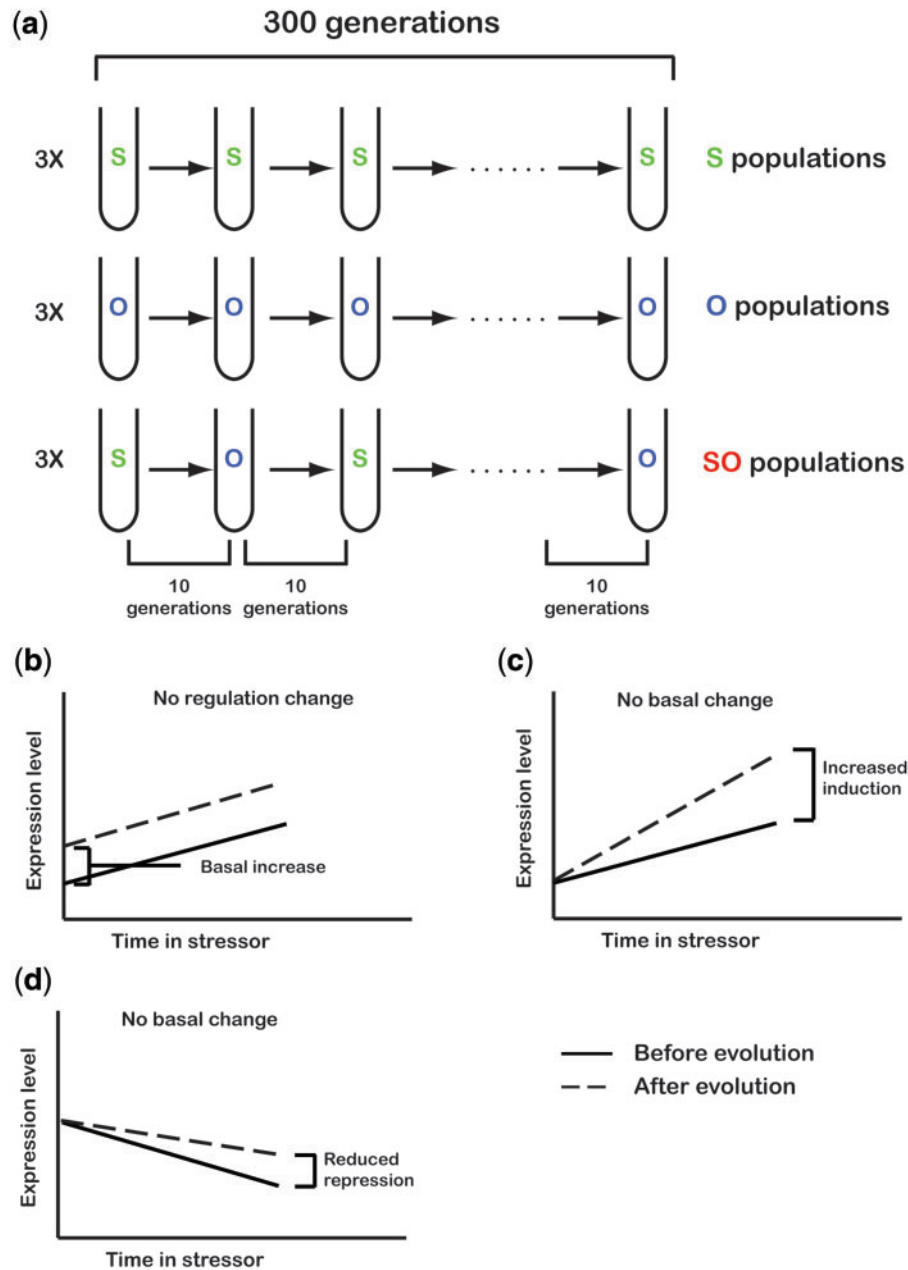
Fig. 1. Experimental design and hypothetical examples of gene expression changes. (*a*) We evolved nine yeast populations in parallel in the laboratory. Three of these populations (SO populations) were exposed to a fluctuating environment, where the cells were first exposed to salt stress for 10 generations and then to oxidative stress for the next 10 generations. We repeated this cycle 15 times for a total of 300 generations. We also evolved six other populations as controls. Three of these populations were continuously exposed to salt stress for 300 generations (S populations), whereas the other three were exposed to continuous oxidative stress for 300 generations (O populations). Panels (*b–d*) show physiological expression changes of a hypothetical gene before (solid line) and after (dotted line) evolutionary adaptation. The *x* axis shows time (on a physiological time scale) after the organism has been exposed to a stressor, and the *y* axis shows the expression level of the gene. (*b*) Basal increase in expression of a gene even in the absence of a stressor. (*c*) Increase in the induction of the gene in the stressor. (*d*) Reduction in repression of the gene in the stressor after evolution. Other changes and combinations thereof are possible (supplementary fig. S2, Supplementary Material online). Expression changes are shown as linear but need not be linear.

A second supporting line of evidence comes from yeast populations evolved in growth medium without stressors, which are referred to as control (C) populations in figure 2. These populations allowed us to completely rule out the possibility that the adaptations in the S, O, and SO populations are solely due to adaptations to the general growth medium or due to the experimental evolution protocol. In salt stress, the S, O, and SO populations had significantly higher fitness than the control populations, suggesting that the adaptations in the S, O, and SO populations are partially specific to salt stress ($w = 1.18 \pm 0.01$ for control populations; $P = 4.1 \times 10^{-5}$ for comparison between S and control populations; $P = 0.004$ for comparison between O and control populations; $P = 4.1 \times 10^{-5}$ for comparison between SO

and control populations; fig. 2a). In oxidative stress, the O and SO populations but not the S populations had significantly higher fitness than the control populations suggesting that the adaptations in O and SO populations are partially oxidative stress specific ($w = 1.05 \pm 0.02$ for control populations; $P = 0.26$ for comparison between S and control populations; $P = 4.1 \times 10^{-5}$ for comparison between O and control populations; $P = 4.1 \times 10^{-5}$ for comparison between SO and control populations; fig. 2b). Taken together, these observations show that evolutionary adaptation has occurred and that part of that adaptation reflects the presence of a stressor in the environment.

Previous studies on the physiological response of yeast and other organisms (e.g., *E. coli* and *Bacillus subtilis*) have shown that organisms have a general stress response that is independent of the stressors that they are exposed to (Gasch et al. 2000; Causton et al. 2001; Petersohn et al. 2001; Weber et al. 2005). Do perhaps all evolutionary adaptations that occurred in our populations affect only this general stress response? This possibility leads to two predictions, neither of which is born out by our data.

First, we would expect that our S and O populations would not differ in fitness in any of the stressors. However, this is not the case. Specifically, consider the S populations, which we evolved under salt stress. Their fitness increased significantly relative to the ancestor in salt stress ($w = 1.28 \pm 0.04$; one sample Wilcoxon signed rank test, $P = 0.004$; fig. 2a), an increase that is significantly higher than the fitness increase in O populations under salt stress (Mann–Whitney U test, $P = 0.003$). Analogously, O populations show a significant fitness increase in oxidative stress (one sample Wilcoxon signed rank test, $P = 0.004$). This increase is also significantly higher than the fitness increase in S populations under oxidative stress (Mann–Whitney U test, $P = 0.0005$). Taken together, these observations suggest that evolutionary adaptation to these stressors does not just affect a general stress response.

A second prediction is that SO populations should not differ at all in their fitness from S populations and from O populations. This prediction is also wrong. The cycling populations increased their fitness under both salt stress ($w = 1.24 \pm 0.05$; Wilcoxon signed rank test, $P = 0.004$) and

Fig. 2. Continued

S populations, which never experienced oxidative stress during this experiment, show a stressor-specific fitness increase under oxidative stress ($w = 1.04 \pm 0.05$ for S populations, $w = 1.13 \pm 0.02$ for O populations, $w = 1.13 \pm 0.04$ for SO populations, and $w = 1.05 \pm 0.02$ for C populations). Again, O and SO populations but not S populations show significantly higher fitness increase compared with the C populations, suggesting that at least part of the adaptations in these populations is due to adaptation to oxidative stress. (c) Relative fitness of the evolved yeast populations in medium without stressors— $w = 1.12 \pm 0.03$ for S populations, $w = 1.09 \pm 0.03$ for O populations, $w = 1.07 \pm 0.02$ for SO populations, and $w = 1.10 \pm 0.01$ for C populations. In each panel, the horizontal line inside a box corresponds to the mean fitness, the height of a box corresponds to the mean ± 1 standard error (SE), and the whisker corresponds to the mean ± 1 standard deviation (SD). An asterisk indicates a significant difference in a pairwise comparison of populations (Mann–Whitney U test).

Fig. 2. Fitness analysis of the evolved populations in salt stress and in oxidative stress. The fitness of each evolved yeast population relative to the ancestral strain was measured with a competition assay against a GFP-tagged reference yeast strain using FACS. A relative fitness of one suggests that the evolved population is as fit as the ancestral strain. (a) Relative fitness of the evolved yeast populations under salt stress. All populations show increased salt-specific fitness in this medium, including O populations and control C populations that never experienced salt stress ($w = 1.28 \pm 0.04$ standard deviations for S populations, $w = 1.22 \pm 0.02$ for O populations, $w = 1.24 \pm 0.05$ for SO populations, and $w = 1.18 \pm 0.01$ for C populations). We note that S, O, and SO populations show a significantly higher fitness increase than the C populations, suggesting salt-specific adaptations in these populations. (b) Relative fitness of the evolved yeast populations under oxidative stress. The O and the SO populations, but not the

oxidative stress ($w = 1.13 \pm 0.04$, $P = 0.004$) compared with the ancestor. Importantly, under oxidative stress, the SO populations had a higher fitness than the S populations ($w = 1.04 \pm 0.05$ for S populations; Mann–Whitney U test $P = 0.004$). Under oxidative stress, their fitness was indistinguishable from that of the O populations, and under salt stress, they also had the same fitness as the S and O populations (fig. 2, Mann–Whitney U tests, $P > 0.19$). In sum, two different lines of evidence argue that part of the evolutionary adaptation we see is partially specific to the stressor applied and does not just affect a general stress response.

Cells Exposed to Oxidative Stress Evolve Cross-Protection against Salt Stress

We next turned to analyzing the changes in gene expression that accompanied evolutionary adaptation.

Although parallel populations in our experiment may well differ in how individual genes change their expression, we focused on common patterns rather than idiosyncratic changes in individual populations. For this reason, we pooled expression data from our replicate populations for statistical analysis.

To interpret our gene expression measurements, it is important to distinguish between two kinds of adaptation, physiological and evolutionary adaptation. Physiological adaptation refers to changes in gene expression within a short time after exposure to a stressor. In contrast, evolutionary adaptation reflects changes in gene expression that occur after long-term exposure to stressors, that is, over many generations. Changes caused by evolutionary adaptation can be further subdivided into "basal" expression changes and changes in "regulation." Basal expression changes are changes that occur even in the absence of stressor in an evolved population relative to the ancestor, whereas regulatory changes arise through changed regulation of genes in response to stressors. Figure 1b–d show several hypothetical examples of the kinds of evolutionary expression changes that can occur in our evolutionary experiments (supplementary fig. S2, Supplementary Material online, contains a more exhaustive list).

Berry and Gasch (2008) showed that the exposure of yeast cells to a primary stressor can prepare them for future secondary stressors that could be different from the primary stressor. We wanted to investigate whether this kind of cross-protection can evolve in our experiment, that is, whether long-term exposure of yeast cells to one stressor can protect these cells against another stressor. We note that cross-protection could be symmetric or asymmetric in nature (fig. 3a). In symmetric cross-protection, adaptation to stressor 1 would protect the cells against stressor 2 ($1 \rightarrow 2$) and vice versa ($2 \rightarrow 1$). In contrast, asymmetric cross-protection would work in only one direction, that is, $1 \rightarrow 2$ or $2 \rightarrow 1$, but not both. In terms of our experiment, asymmetric cross-protection means that evolutionary adaptation to salt stress could protect cells against oxidative stress, whereas evolutionary adaptation to oxidative stress might not protect cells against salt stress or vice versa.

Two lines of evidence from our experiments point to evolution of asymmetric cross-protection, where long-term adaptation to oxidative stress protects yeast cells against salt stress but not vice versa. First, the O populations show a significant fitness increase in salt compared with the ancestor ($w = 1.22 \pm 0.02$; $P = 0.004$), although they were never exposed to salt stress during our evolution experiment (fig. 2a). Their fitness in salt is also significantly higher than their fitness in medium without stressor (Mann–Whitney U test, $P = 4 \times 10^{-5}$). In contrast, although the S populations show a (small but significant) fitness increase under oxidative stress relative to the ancestor ($w = 1.04 \pm 0.05$; $P = 0.008$), their fitness in oxidative stress is lower than in medium without stressor (Mann–Whitney U test, $P = 0.008$; fig. 2b and c). Thus, this fitness increase in the S populations does not reflect an adaptation to the oxidative stressor in the growth medium. These observations suggest that previous exposure to oxidative stress protects cells against salt stress but not vice versa. The second line of evidence comes from fitness measurements that we carried out in two different cycles of exposure to stressors. Briefly, SO and O populations show higher fitness in the O $\rightarrow$ S cycle than in the S $\rightarrow$ O cycle, suggesting evolution of asymmetric cross-protection (supplementary results S1, Supplementary Material online).

Both the O and the SO populations had been exposed to oxidative stress for prolonged periods during the evolution experiment, one of them continuously and the other multiple times. This raised the question whether such prolonged exposure is a requirement for the cross-protection we see. In other words, is the cross-protection we observed an evolutionary response to prolonged or repeated oxidative stress, or is it independent of such prolonged exposure? Our experiments allow us to distinguish between these possibilities, because the S populations were not exposed to oxidative stress during the evolution experiment. If cross-protection is an evolved feature, we would predict that it does not occur in S populations. Indeed, in these populations, there is no fitness difference between the O $\rightarrow$ S cycle (fig. 4a, left-most data) and the S $\rightarrow$ O cycle (fig. 4b; left-most data) (Mann–Whitney U test, $P = 0.1043$). That is, in populations not exposed to oxidative stress during the evolution experiment, pre-exposure to oxidative stress does not increase fitness. In sum, our observations suggest that our populations evolved asymmetric cross-protection, where pre-exposure to oxidative stress can protect a population against salt stress.

## Evolved Asymmetric Cross-Protection Is Reflected in Shared Genes That Change Regulation

We next asked whether the evolved cross-protection we observed had also left an evolutionary signature in gene expression changes. To find out, we turned our attention to genes that changed their regulation during our evolution experiment (see Materials and Methods). The observations we made regard quantitative differences in the number of genes that change regulation in the S population, on the one hand, and in the O and SO populations, on the other hand. We first turn to the S populations, where 61 genes changed their
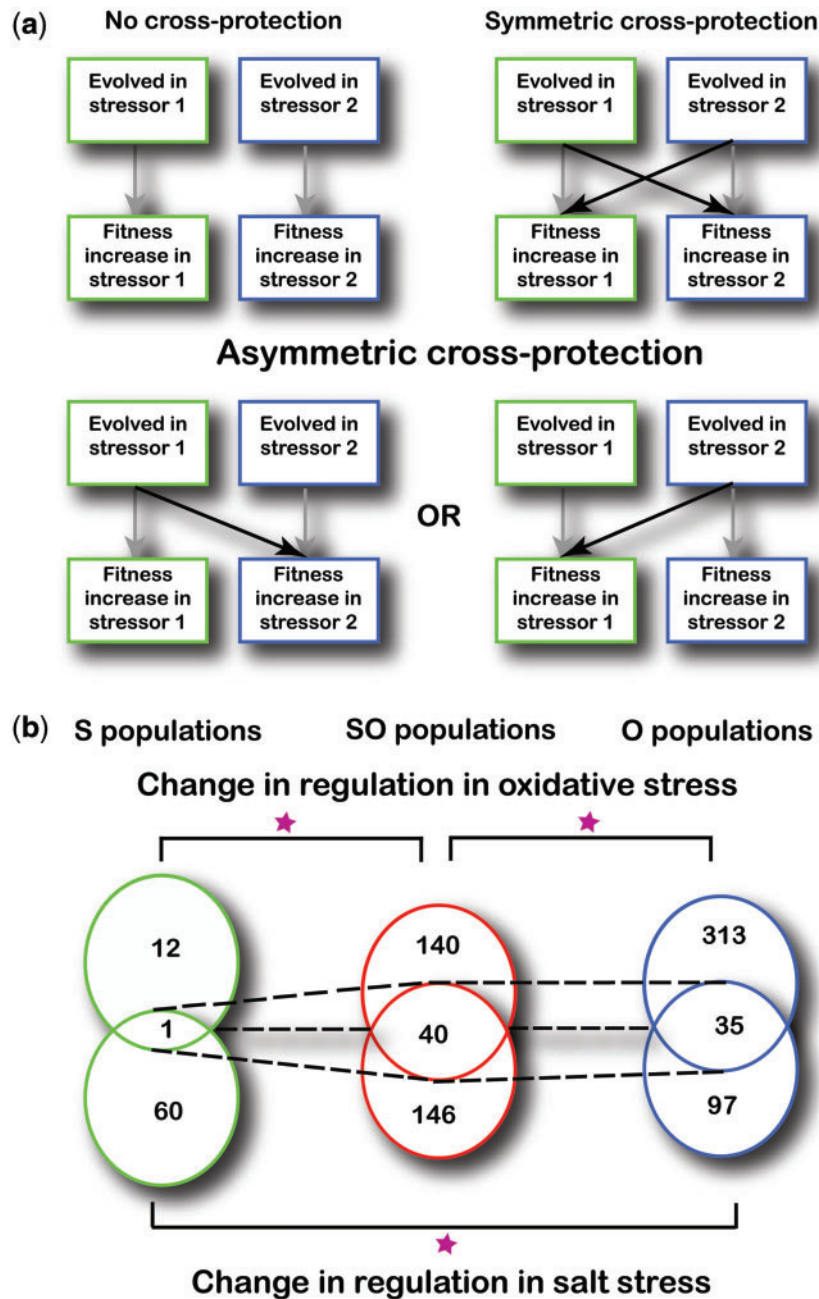
Fig. 3. Symmetric and asymmetric cross-protection. (*a*) The types of stress cross-protection that can occur in our experiments. Symmetric cross-protection means that adaptation to stressor 1 protects the cells against stressor 2 and vice versa. In contrast, in case of asymmetric cross-protection, adaptation to only one stressor protects against the other but not vice versa. Asymmetric cross-protection could be of two different types, as shown. (*b*) Venn diagram showing overlap between genes with changed regulation in oxidative stress (upper three numbers) and in salt stress (lower three numbers). The middle three numbers, in the intersection of the ellipses, correspond to genes that change regulation both in oxidative stress and in salt stress. For example, in S populations, 13 and 61 genes show changes in regulation in oxidative stress and in salt stress, respectively, with only one gene that is common between them. See text for details. The purple asterisks indicate significant difference in overlap between different populations.

regulation relative to the ancestral strain in the salt-evolved S populations—when the S populations are exposed to salt (fig. 3*b*, left-most data). Many fewer genes (13 genes) changed their regulation in the S populations when these populations are exposed to oxidative stress. Importantly, the intersection of these two sets of genes is very small: only one gene (1.64% of 61 genes) changed regulation in response to both salt and

oxidative stress (fig. 3*b*, left-most data). We tested the null hypothesis that this overlap could occur by chance alone, that is, for sets of 61 and 13 genes drawn at random from the yeast genome (supplementary methods S1, Supplementary Material online). The answer is yes (randomization test $P = 0.131$; supplementary methods S1, Supplementary Material online).

(a) **Relative fitness in the S→O cycle**

(b) **Relative fitness in the O→S cycle**
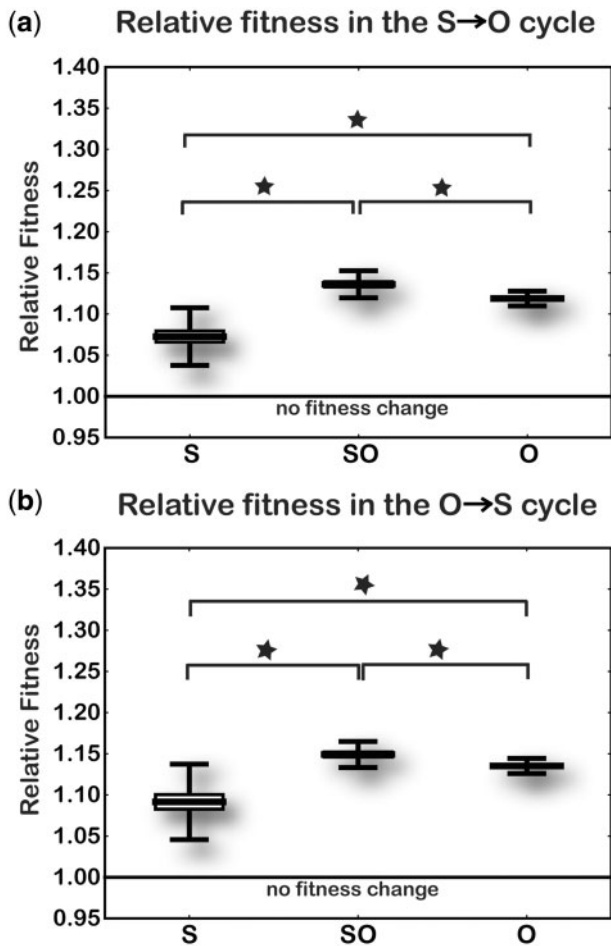
FIG. 4. Fitness analysis of the evolved populations in one S → O transition and in one O → S transition. (*a*) Relative fitness in the S → O transition. In these competition assays, we first let yeast cells grow for 24 h under salt stress, then for another 24 h under oxidative stress, and measured competitive fitness thereafter ($w = 1.07 \pm 0.04$ for S populations, $w = 1.12 \pm 0.01$ for O populations, and $w = 1.14 \pm 0.02$ for SO populations). (*b*) Relative fitness in the O → S transition. As in (*a*), except that cells were first exposed to oxidative stress and then to salt stress ($w = 1.09 \pm 0.05$ for S populations, $w = 1.14 \pm 0.01$ for O populations and $w = 1.15 \pm 0.02$ for SO populations). For O populations and SO populations, the fitness increase is significantly higher in the O → S transition compared with the fitness in the S → O transition ($P = 2.37 \times 10^{-8}$ for O populations and $P = 0.001$ for SO populations). However, this does not hold for S populations ($P = 0.1043$). The line in the box represents the mean fitness, the box represents mean ± 1 standard error (SE), and the whisker represents mean ± 1 standard deviation (SD). The asterisks indicate a significant difference in fitness (Mann–Whitney *U* test).

This overlap is much greater in O populations (fig. 3*b*, right-most data). Analogously to what we observed in the S populations, more genes change their regulation when O populations are exposed to oxidative stress (348 genes) than when they are exposed to salt stress (132). However, now the overlap between these two sets of genes (35 genes or 10.06% of 348 genes) is greater than expected by chance alone (randomization test $P < 10^{-7}$). In addition, the overlap is also significantly greater than in the salt-evolved populations (χ^2 test $P = 0.0392$, df = 1, supplementary methods S2,

Supplementary Material online). We note that this significant overlap does not just result from the greater number of genes that change their regulation in O than in S populations (fig. 3*b*, right-most data and left-most data), because our tests account for this fact (supplementary methods S1, Supplementary Material online). Among these 35 genes, there are two genes that show a change in induction in salt and in peroxide. One gene, *PUT4*, was induced in response to osmotic stress in three previous physiological studies of the stress response (Posas et al. 2000; Rep et al. 2000; Yale and Bohnert 2001). The second gene, *YDL199C*, encodes a putative transporter protein that has been shown to reduce oxidative stress resistance when knocked out (Higgins et al. 2002). In addition, O populations harbor 33 genes that show change in repression in salt and in peroxide (supplementary results S3, Supplementary Material online).

An analogous pattern holds in SO populations (fig. 3*b*, middle data). Here, the overlap between the set of genes that changed regulation in salt and oxidative stress (40 genes) is greater than expected by chance alone (randomization test $P < 10^{-7}$). It is also significantly greater than the overlap in S populations (χ^2 test $P = 0.0045$, df = 1). One of the affected genes, *PUT4*, is shared with O populations. A second gene, *DDR48*, was found to be upregulated in salt and in peroxide in previous studies of physiological stress adaptation (Gasch et al. 2000; Posas et al. 2000; Rep et al. 2000; Causton et al. 2001; Yale and Bohnert 2001). Yet another gene, *YOR152C*, is upregulated as part of the common environmental stress response (Gasch et al. 2000) (supplementary results S3, Supplementary Material online).

We note parenthetically that in the SO populations, the number of genes that change in regulation is almost equal between salt stress (186 genes) and oxidative stress (180 genes). These two numbers are significantly more similar to each other than in the S and O populations (χ^2 test at df = 1, $P < 0.0001$ both for comparison between S and SO populations and between O and SO populations; supplementary methods S3, Supplementary Material online), which is consistent with the fact that these populations have been evolved in both stressors for an equal amount of time.

In sum, asymmetric cross-protection is associated with a significantly increased fraction of genes that change their expression in both salt and oxidative stress, when populations evolve under sustained (O populations) or repeated (SO populations) oxidative stress.

In the supplementary material, Supplementary Material online, we discuss in more detail the numbers of genes that change either basal expression or regulation in S, O, and SO populations and the genes with expression changes that are shared between these populations (supplementary fig. S3 and supplementary results S2, Supplementary Material online). Also in the supplementary material, Supplementary Material online, we report another observation that is consistent with evolved asymmetric cross-protection. Specifically, genes changing their expression evolutionarily in O and SO populations are significantly more similar in their function to each other than to genes that change their expression in S populations (supplementary fig. S4 and supplementary

results S4, Supplementary Material online). We also associate genes that change regulation in salt stress and in oxidative stress in these populations with phenotype data for null, reduction in function, and overexpression mutants from *Saccharomyces* Genome Database (supplementary fig. S13, Supplementary Material online) (phentoype_data.tab from http://www.yeastgenome.org/download-data/curation/, last accessed November 19, 2012; Cherry et al. 2012).

Evolved Anticipation of Stressors in SO Populations

We next asked whether asymmetric cross-protection could explain all the fitness increase we observe in SO populations. If this was so, the fitness of O populations and SO populations would be identical under cycling conditions. However, this is not the case. Even though the fitness of O and SO populations does not differ significantly in salt stress ($P = 0.1903$) or in oxidative stress ($P = 0.5457$), fitness is higher in SO populations both in the O → S cycle (fig. 4a, $P = 0.001$) and in the S → O cycle (fig. 4b, $P = 9 \times 10^{-10}$). This higher fitness could have at least two nonexclusive explanations.

First, the SO populations may have evolved adaptations unique to them. In the supplementary material, Supplementary Material online, we show that our gene expression data support this possibility: In the SO populations, but not in the S or O populations, a distinct group of genes has changed their basal expression in the course of the experiment (supplementary results S5 and supplementary figs. S5 and S6, Supplementary Material online). These include genes well known to be involved in the physiological stress response, such as the gene *FRT2*, which can promote growth in conditions of high Na$^+$ concentrations (Heath et al. 2004), and the genes *FRE7* and *HMX1*. Knockout mutations in these genes can reduce oxidative stress resistance (Higgins et al. 2002; Collinson et al. 2011).

Second, the cycling (SO) populations may have acquired the ability to anticipate the next stressor in each cycle. Adapting physiologically to that stressor before it arises, for example, through gene expression changes, might help explain the higher fitness we observe in the cycling populations. This possibility gives rise to several predictions about changes in gene regulation that we tested next.

The SO population shows evolutionary adaptation in the regulation of two sets of genes, one upon exposure to stressor S (S-specific genes) and the other upon exposure to stressor O (O-specific genes). In this population, S-specific genes would change their expression after exposure to S, that is, as a physiological adaptation to S. If the appearance of stressor S also served as an anticipatory signal for stressor O, one would expect that stressor S also triggers the expression of many O-specific genes. More specifically, in population SO, the number of these O-specific genes should be greater than in a population that was only exposed to stressor S during its evolution. If so, one could conclude that the population has acquired the ability to "interpret" stressor S as anticipating stressor O. Conversely, when population SO is exposed to stressor O, it would show changes in the expression of O-specific genes. If it also shows changes in the expression

of many S-specific genes, one might conclude that stressor O can help the population anticipate stressor S. These simple considerations show that anticipation can be symmetric or asymmetric (fig. 5a; Mitchell et al. 2009). Symmetric anticipation means that each stressor serves as a signal to anticipate the other stressor. Asymmetric anticipation means that only one of the stressor can help anticipate the other stressor.

The scenario from the preceding paragraph leads to three specific predictions. First, in the SO populations, the sets of genes that evolved changed regulation in response to S and O should show a statistically significant overlap, and, more importantly, this overlap should be greater than in the S and O populations that were never exposed to the fluctuating environment, otherwise we could not conclude that the overlap is an evolved response to the fluctuating environment. We already discussed earlier that the sets of genes with regulatory changes in both salt stress and oxidative stress overlap to a significantly greater extent in SO populations than in S populations (fig. 3b, χ^2 test $P = 0.0045$, df = 1). The overlap is also significantly higher than in O populations (χ^2 test $P = 0.0046$, df = 1), thus confirming the prediction.

A second prediction is that the overlap of the sets of genes changing expression in physiological response to both salt and oxidative stress should be higher in the SO populations than in the ancestor. This is indeed the case (χ^2 test, $P < 0.0001$, df = 1, supplementary fig. S7, Supplementary Material online). Also, the overlap of these sets of genes is significantly higher in the SO populations when compared with the S populations (χ^2 test, $P < 0.0001$, df = 1), but not when compared with the O populations (χ^2 test, $P = 0.70$, df = 1) (supplementary fig. S7, Supplementary Material online). We discuss the latter observation further in the supplementary results S6, Supplementary Material online.

We note that these two predictions do not distinguish between symmetric or asymmetric anticipation. However, a third prediction does. It is illustrated by the schematic of figure 5b. Let us denote by A_1 the fraction of genes that have changed regulation both after exposure to salt in SO populations and after exposure to oxidative stress in O populations. If salt has become an anticipatory signal for oxidative stress in SO populations, then A_1 should be significantly greater than A_2 (fig. 5b), which denotes the fraction of genes that change regulation both after exposure to salt stress in S populations and to oxidative stress in O populations. This is indeed the case (χ^2 test, $P < 0.0001$, df = 1). In terms of absolute numbers, there are 45 genes in set A_1, that is, genes that changed regulation in response to salt stress in SO populations and that changed regulation in response to oxidative stress in O populations. There are, however, only 12 genes in set A_2, that is, genes with changed regulation in O populations in response to oxidative stress and with changed regulation in S populations in response to salt stress.

Conversely, if oxidative stress has become a signal in SO populations to anticipate the salt stress response, then A_3 (fig. 5b) should be significantly greater than A_2. That is, the fraction of genes that change their expression when exposed
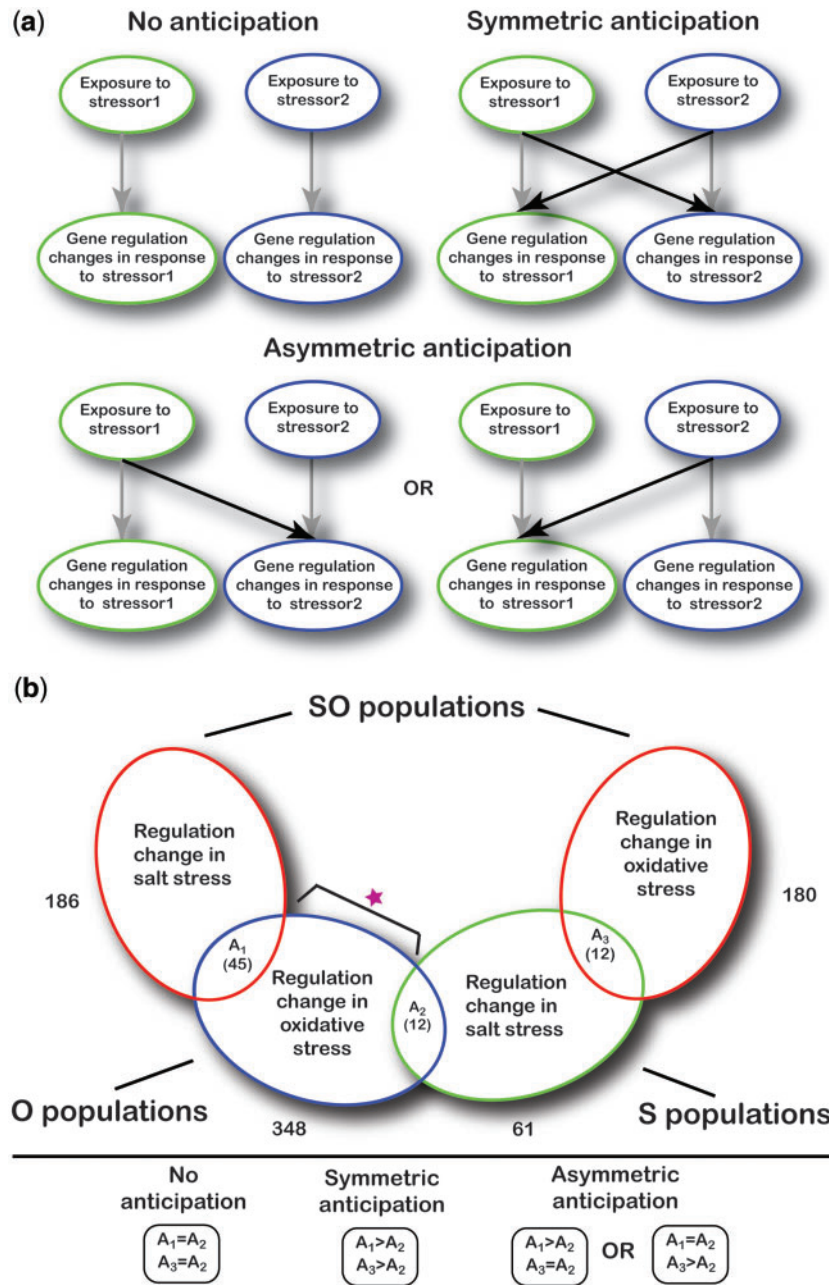
Fig. 5. Evolved symmetric and asymmetric anticipation and its detection in the SO populations. (*a*) The concepts of symmetric and asymmetric anticipation. Symmetric anticipation means that each stressor serves as a signal to anticipate the other stressor. Asymmetric anticipation means that only one of the stressors can help anticipate the other stressor. (*b*) Criteria and data to detecting anticipation. A_1 is the fraction of genes with changed regulation in salt stress in SO populations and in oxidative stress in O populations; A_2 is the fraction of genes with changed regulation both in salt stress in S populations and in oxidative stress in O populations; and A_3 is the fraction of genes with changed regulation both in salt stress in S populations and to oxidative stress in SO populations. The criteria for evidence for no anticipation, symmetric anticipation, and asymmetric anticipation are summarized below the Venn diagram. In our experiments, we found that the set A_1 contains 45 genes and is significantly greater than set A_2, which contains 12 genes. Set A_3 contains 12 genes, a number that does not differ significantly from the size of set A_2. The purple asterisk indicates a significant difference in the sizes of the sets A_1 and A_2.

to oxidative stress in SO populations, and that also change their expression in response to salt stress in S populations, should be significantly greater than the fraction of genes that change regulation both after exposure to salt stress in S populations and to oxidative stress in O populations. This is not the case (χ^2 test, $P = 0.05$, df = 1). The absence of evidence for oxidative stress as an anticipatory signal for salt stress could be

due to the fact that one cannot completely disentangle the effects of cross-protection and anticipation (see Discussion).

In sum, three lines of evidence based on gene expression changes in our evolved populations suggested that asymmetric anticipatory regulation evolved in our lines, where salt stress can help anticipate oxidative stress, but not vice versa. The genes most likely to be affected by this anticipatory

regulation are genes that are contained in set A_1 but not in set A_2 (fig. 5*b*). There are 33 such genes (supplementary table S1, Supplementary Material online). Three of them have a known role in the stress response. Specifically, a null mutant of gene *YGR035C* shows decreased hyperosmotic stress resistance (Yoshikawa et al. 2009), and the genes *SPS4* and *HXT2*, are associated with tolerance of and adaptation to salt in yeast (Warringer et al. 2003).

Discussion

All three kinds of populations (S, O, and SO) that we studied adapted evolutionarily to their respective stressors within a mere 300 generations and experienced a fitness increase between 10% and 30% relative to the ancestor. Fitness differences between the populations indicate that their evolutionary adaptation is not due to a common stress response but at least partly specific to salt or oxidative stress. Our data also speak to the possibility of a trade-off in fitness, which would occur if the elevated fitness of S populations comes at the price of a reduced fitness under oxidative stress and vice versa for O populations. However, no such trade-off is evident.
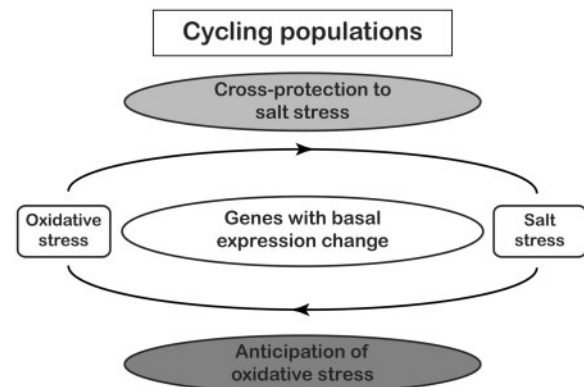
We observed the evolution of asymmetric cross-protection (fig. 2*a* and *b*), where exposure to oxidative stress protected the cells against salt stress but not vice versa. This assessment is based on fitness differences of populations exposed to oxidative stress and is accompanied by changes in gene regulation in the evolved populations. Specifically, the sets of genes that changed their regulation in response to oxidative and salt stress share many more genes in populations where cross-protection arose than in the ancestor. We emphasize that cross-protection is an evolutionary adaptation that occurred during our experiment, and that depends on prolonged exposure to oxidative stress, because our S populations do not show it. We note that the cross-protection evolved not only in the cycling SO populations but also in the O populations.

One question that remains unanswered is why long-term adaptation to oxidative stress protects yeast cells against salt stress but not vice versa. We discuss two candidate explanations here. First, it is possible that our O populations took an evolutionary path for adaptation to oxidative stress that also partially overlaps with the evolutionary path of adaptation to salt stress. This idea is supported by the fact that similar functional gene classes are significantly enriched or impoverished for genes with regulation change in peroxide in O populations and for genes with regulation change in salt in S populations (supplementary fig. S6, Supplementary Material online). A second possible explanation comes from the observation that many environmental stressors induce the generation or accumulation of reactive oxygen species inside cells, thereby causing oxidative stress. Studies from other yeast species and plants suggest that some oxidative stress response genes might also be associated with the osmotic stress response (Gossett et al. 1996; Cazalé et al. 1998; Lin and Kao 2002; Chao et al. 2009). Thus, long-term adaptation to oxidative stress might partially alleviate the harmful effects of reactive oxygen species caused by other stressors.

A related phenomenon has also been observed for bacterial antibiotic resistance, where many antibiotics work through generating reactive oxygen species in the cells (Albesa et al. 2004; Dwyer et al. 2007; Hassett and Imlay 2007; Kohanski et al. 2007). Thus, adaptations to oxidative stress or induction of oxidative stress response pathways through cellular signaling molecules help bacteria survive these antibiotics (Dwyer et al. 2009; Lee and Collins 2011; Poole 2012; Vega et al. 2012).

Cross-protection alone cannot explain all the fitness increase in the SO populations in our experiment, because the cycling populations have significantly higher fitness than the O populations under cycling conditions. We investigated two potential nonexclusive causes for this observation through their gene expression signatures. First, the SO populations may have experienced adaptations unique to them, which may include unique changes in gene expression or regulation. We showed that candidate genes with such changes indeed exist. Specifically, many genes changed their basal expression specifically in SO populations (supplementary results S5 and supplementary figs. S5 and S6, Supplementary Material online). Second, the SO populations may have "learned" about the periodic nature of the environmental change and become able to anticipate it. Three lines of evidence for this possibility exist through the proportion of genes that change expression in the SO populations, compared with the S, O, and ancestral populations (figs. 3*b* and 5*b* and supplementary fig. S7 and supplementary results S6, Supplementary Material online). More specifically, this evidence argues for asymmetric anticipation, where salt stress helps anticipate oxidative stress, but not vice versa, in SO populations. Figure 6 summarizes our observations schematically.

The evolutionary adaptations we characterize are complex and involve many genes. It is thus not surprising that our observations leave open questions. First, we have not been

Cycling populations

Cross-protection to salt stress

Oxidative stress — Genes with basal expression change — Salt stress

Anticipation of oxidative stress

Fig. 6. Summary of observations for cycling populations. The rounded boxes correspond to the two different stressors between which the SO populations cycle (arrows). Fitness and gene expression data provided evidence for evolved cross-protection of oxidative stress against salt stress (light gray ellipse). Gene expression data also indicated asymmetric anticipation of oxidative stress by salt stress (dark gray ellipse) and basal expression changes in genes that were unique to cycling populations (white ellipse). Which of these three features affect fitness most strongly cannot be distinguished from our experiments.

able to disentangle the effects of cross-protection and anticipation on fitness in the cycling SO populations. Doing so may in general be very difficult, for the following reason. In cross-protection, a physiological state attained in one stressor protects against a second stressor. In comparison, in anticipation, exposure to the first stressor is necessary for triggering a second protective physiological state. To distinguish between cross-protection and anticipation, it is thus essential to know whether a cell's physiological state when exposed to the second stressor is a result of physiological adaptation to the first stressor or whether it is merely triggered by exposure to the first stressor but specifically protects against the second stressor. Because physiological states may change almost immediately after any environmental change (Gasch et al. 2000; Causton et al. 2001), it is difficult to make this distinction.

Although our experiments did not allow us to clearly separate cross-protection and anticipation, they revealed specific gene expression signatures that point toward anticipatory regulation in the cycling SO populations. The fitness changes we observed point in the same direction, because the SO populations have significantly higher fitness than the O populations both in the O → S and in the S → O transition. Using control populations is crucial in this regard, because in their absence, one might falsely attribute the adaptations we see in cycling populations solely to anticipation, instead of to a combination of cross-protection and anticipation.

A second, related limitation is that we do not know whether cross-protection, anticipation, or basal expression changes increase fitness more strongly. The reason is again the inability to completely separate anticipation and cross-protections in cycling populations. However, the data hint that cross-protection has the greatest effect on fitness, because the cycling populations have higher fitness in the O → S transition than in the S → O transition (fig. 4a and b). A third open question is why cross-protection and anticipation are asymmetric and why they occur in opposite directions.

Multiple genes that changed their regulation and expression in our experiments have been previously implicated in physiological adaptation to salt or oxidative stress (supplementary results S3 and S5, Supplementary Material online). It may be tempting to search for single genes that may be causally responsible for the adaptations we see. However, two lines of evidence suggest that such a search may not be successful. Recent work has shown (Berry et al. 2011) that the genes required for physiological adaptation to a stressor do not only depend on the stressor but also on other stressors that preceded it. Such interdependencies make the identification of single genes that are unconditionally responsive to a stressor difficult. Second, both the physiological stress response and evolutionary adaptations to stress involves many genes. For example, evolutionary adaptations to salt stress are complex, polygenic, and do not involve measurable allele frequency increases in single adaptive alleles, at least on the time scale of a laboratory evolution experiment (Dhar et al. 2011).

In sum, past work on physiological adaptation has demonstrated the existence of both cross-protection and anticipation (Völker et al. 1992; Cullum et al. 2001; Greenacre and

Brocklehurst 2006; Schild et al. 2007; Berry and Gasch 2008; Tagkopoulos et al. 2008; Wolf et al. 2008; Mitchell et al. 2009; Rodaki et al. 2009; Berry et al. 2011). Our work shows that both phenomena, as well as relevant basal gene expression changes, can evolve within a mere 300 generations of laboratory evolution. Future work may be needed to show which of these changes are more important in the adaptation of organisms to varying environments.

Materials and Methods

Strains and Media

We used the haploid yeast strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; ATCC#201388) in this study. We started all laboratory evolution experiments from the same clone of BY4741, which we refer to as the "ancestral" strain. We refer to populations derived from the ancestral strain through serial transfer cycles as "evolved" populations. We refer to the three replicate yeast populations evolved in salt as S populations, three replicate populations evolved in hydrogen peroxide stress as O populations, and three replicate populations evolved in periodically occurring salt stress and oxidative stress as SO populations. We also evolved three replicate control (C) populations in general growth medium without salt stress and oxidative stress. We estimated the growth rates of the evolved populations and that of the ancestral strain relative to a BY4741 strain in which the CWP2 gene is tagged with GFP. We refer to this GFP-tagged strain as the "reference" strain. We obtained ancestral and reference strains from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/yeast.html, last accessed November 19, 2012) and Invitrogen (www.invitrogen.com, last accessed November 19, 2012, and http://yeastgfp.yeastgenome.org/, last accessed November 19, 2012) (Ghaemmaghami et al. 2003), respectively. For serial transfers, we cultured cells in YP (consisting of 2% peptone and 1% yeast extract) and 2% galactose (YPG), YPG supplemented with 0.5 M NaCl (YPGS), and YPG supplemented with 1 mM H_2O_2 (YPGO). We supplemented all media for serial transfer with 50 μg/ml ampicillin and 25 μg/ml tetracycline to minimize the risk of contamination. We verified the stability of hydrogen peroxide in YPG medium using a Merckoquant peroxide test kit (Merck Catalog No. 1100810001), which showed that after 24 h at 30°C with shaking, the concentration of hydrogen peroxide in YPG medium still exceeded 80% of the initial hydrogen peroxide concentration.

We chose the concentrations of salt and hydrogen peroxide based on growth rate and viability measurements of ancestral yeast strain in these stressors (supplementary figs. S11 and S12, Supplementary Material online). Briefly, we found that salt stress from 0.5 M NaCl reduces the growth rate of yeast, so that the final cell number after 24 h of growth is approximately one-third of the cell number without stressors, but it does not affect the viability of yeast cells even after growth for 24 h. In contrast, under oxidative stress from 1 mM peroxide, the final cell number after 24 h of growth is approximately 87% of the cell number without stressor. However, oxidative stress at 1 mM peroxide leads to a loss

in cell viability, and higher peroxide concentration causes a population size reduction greater than 90%, when the stressor is applied only for 3 h (supplementary fig. S12, Supplementary Material online).

Serial Transfer

We started nine parallel serial transfer experiments with an overnight culture derived from one single clone of the ancestral strain. In each parallel experiment, we grew 50 ml of yeast culture in an incubating shaker for 24 h at 220 revolutions per minute (rpm) and 30°C, after which cultures had reached stationary phase. Every 24 h, we transferred 50 μl of stationary culture into 50 ml of fresh culture medium. We carried out 30 such transfer cycles for a total of approximately 300 generations (each transfer cycle involved approximately $\log_2 1{,}000 \approx 10$ cell generations). In three of the parallel experiments, we used YPGS medium for each transfer; in three other parallel experiments, we used YPGO medium; and in another three experiments, we used YPGS and YPGO medium alternatively. Before each transfer, we froze 1.5 ml of culture supplemented with 25% glycerol and stored this cell suspension at −80°C for future analysis. We examined cultures periodically for contamination by microscope and through plating.

Competition Assays

To compare growth rates of the evolved populations with that of the ancestral strain, we performed competition assays using fluorescence-activated cell sorting (FACS), as described later. We grew cells overnight from frozen glycerol stocks in 4 ml YPD medium (30°C, 220 rpm) until they had reached late logarithmic phase ($<1.5 \times 10^8$ cells/ml). To estimate cell counts, we used a Neubauer cytometer. In each culture, we then adjusted the cell numbers to 2.5×10^7 cells/ml with YPD. These preculturing steps ensured that cells were in comparable physiological states with high viability before competition. For the competition assay, we mixed equal cell numbers (2.5×10^6) of the reference strain and of the competing strain in 10 ml YPGS or YPGO medium (5.0×10^5 ml^{-1} final cell density). We collected a 2.0 ml aliquot of this culture, centrifuged it, and resuspended the cells in 1.0 ml PBSE (Phosphate Buffer Saline EDTA) buffer (10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, 137 mM NaCl, 2.7 mM KCl, 0.1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4). We stored these cells overnight at 4°C. We used FACS to measure the relative cell numbers $N_r(0)$ of the reference strain and $N_e(0)$ of an evolved population (or $N_a(0)$ of the ancestral strain, depending on the experiment) at the beginning of the competition assay. We carried out all the competition assays in three replicates. For each replicate, we grew up 2 ml cell aliquots for 24 h in an incubating shaker at 220 rpm and 30°C. At the end of 24 h, we counted the relative cell numbers $N_r(1)$ and $N_e(1)$ (or $N_a(1)$) using FACS with a Beckman Coulter Cytomics FC500 fluorescence-activated cell sorter. The histogram of fluorescing and nonfluorescing cells provided the relative number of cells of the reference strain (GFP tagged) and the competing strain (evolved population or ancestral strain).

For measurement of fitness in a change from oxidative stress to salt stress (O → S), we first grew cells subject to oxidative stress, that is, in YPGO medium following the same protocol as earlier (24 h, 220 rpm, 30°C). We then diluted the cells 1,000 times into fresh YPGS medium and grew them for another 24 h (220 rpm, 30°C). We then counted the relative cell numbers for the reference strain ($N_r(2)$) and the evolved ($N_e(2)$) or ancestral strain ($N_a(2)$), depending on the experiment, using same FACS protocol as described earlier. Fitness measurement in the S → O transition proceeded exactly analogously, except that cells were first grown in YPGS for 24 h and then in YPGO for another 24 h.

Estimating Growth Rate Differences

From the competition assays, we determined the growth rate differences as follows. If the reference strains R and some other strain E grow according to $N_r(t) = e^{r_r t} N_r(0)$ and $N_e(t) = e^{r_e t} N_e(0)$, where r_r and r_e are strain-specific growth rates, and where $N_i(t)$ are population sizes of the respective strains at time t, then

$$\frac{N_e(t)}{N_r(t)} = \exp[(r_e - r_r)t]\frac{N_e(0)}{N_r(0)} = \exp[(m_e - m_r)t]\frac{N_e(0)}{N_r(0)}.$$

Here, m_e is the Malthusian fitness of strain E and m_r is the Malthusian fitness of strain R. Because we measured population numbers through FACS after $t = 1$ day of competition, we estimated m_e (dimension $[d^{-1}]$) as follows:

$$m_e - m_r = \ln\left(\frac{N_e(1)/N_r(1)}{N_e(0)/N_r(0)}\right) = \ln\left(\frac{[100 - FL_e(1)]/FL_e(1)}{[100 - FL_e(0)]/FL_e(0)}\right),$$

where $FL_e(0)$ represents the percentage of GFP-tagged reference cells in the medium at the beginning of the competition assay, and $FL_e(1)$ represents the percentage of these cells after 1 day of competition. Similarly,

$$m_a - m_r = \ln\left(\frac{N_a(1)/N_r(1)}{N_a(0)/N_r(0)}\right) = \ln\left(\frac{[100 - FL_a(1)]/FL_a(1)}{[100 - FL_a(0)]/FL_a(0)}\right).$$

To compare the cell numbers of an evolved population to that of the ancestral strain, we calculated the dimensionless ratio $N = N_e/N_a$. This ratio is equivalent to

$$N = \frac{N_e}{N_a} = \left(\frac{N_e(1)/N_e(0)}{N_a(1)/N_a(0)}\right) = \exp(r_e - r_a) = \exp(m_e - m_a).$$

Therefore,

$$\begin{aligned}
m_e - m_a &= \ln\left(\frac{N_e}{N_a}\right) = \ln\left(\frac{N_e(1)/N_e(0)}{N_a(1)/N_a(0)}\right)\\
&= \ln\left(\frac{[100 - FL_e(1)]/FL_e(1)}{[100 - FL_e(0)]/FL_e(0)}\right)\\
&\quad - \ln\left(\frac{[100 - FL_a(1)]/FL_a(1)}{[100 - FL_a(0)]/FL_a(0)}\right).
\end{aligned}$$

Because the growth of our yeast cells is density dependent and is limited to a 1,000-fold increase per culture transfer cycle, we can estimate m_a as $m_a = m_{av} = \ln(1{,}000)/(1 \, d) = 6.9078 \, d^{-1}$ (where m_{av} is the average Malthusian fitness for

the ancestral strain, as estimated from this 1,000-fold increase). We define the selection coefficient s as the difference in Darwinian fitness between an evolved population and the ancestral strain, that is, $s = (m_e - m_a)/m_a = w - 1$ (Lenski et al. 1991), where $s > 0$ (or $w > 1$) indicates that the evolved population has an advantage over the ancestral strain.

For measurement of fitness in the O → S and S → O transitions, we have

$$\frac{N_e(1)}{N_r(1)} = \exp[(r_e - r_r)]\frac{N_e(0)}{N_r(0)} = \exp[(m_e - m_r)]\frac{N_e(0)}{N_r(0)}$$

and

$$\frac{N_e(2)}{N_r(2)} = \exp[(r_e - r_r)]\frac{N_e(1)/1{,}000}{N_r(1)/1{,}000} = \exp[(m_e - m_r)]\frac{N_e(1)}{N_r(1)}.$$

This yields

$$\frac{N_e(2)}{N_r(2)} = \exp[(m_e - m_r)]\frac{N_e(1)}{N_r(1)} = \exp[2(m_e - m_r)]\frac{N_e(0)}{N_r(0)}.$$

Thus, following a similar derivation as that above,

$$N = \frac{N_e}{N_a} = \left(\frac{N_e(2)/N_e(0)}{N_a(2)/N_a(0)}\right) = \exp[2(m_e - m_a)].$$

Therefore,

$$
\begin{aligned}
m_e - m_a &= \frac{1}{2}\ln\left(\frac{N_e}{N_a}\right) = \frac{1}{2}\ln\left(\frac{N_e(2)/N_e(0)}{N_a(2)/N_a(0)}\right) \\
&= \frac{1}{2}\ln\left(\frac{[100 - FL_e(2)]/FL_e(2)}{[100 - FL_e(0)]/FL_e(0)}\right) \\
&\quad - \frac{1}{2}\ln\left(\frac{[100 - FL_a(2)]/FL_a(2)}{[100 - FL_a(0)]/FL_a(0)}\right) \\
&\quad\left(\text{dimension}\left[d^{-1}\right]\right).
\end{aligned}
$$

As above, $m_a = m_{av}{}^* = \ln(1{,}000^2)/(2\,d) = 2{*}6.9078/2\,d^{-1} = 6.9078\,d^{-1}$, because there is a 10^6-fold increase in the number of cells over two cycles, that is, in 2 days. These considerations yield

$$s = (m_e - m_a)/m_a = w - 1 = \frac{\frac{1}{2}\ln\left(\frac{N_e(2)/N_e(0)}{N_a(2)/N_a(0)}\right)}{6.908}.$$

Whole-Genome Transcriptome Analysis

We analyzed the mRNA expression levels in the ancestral strain and in the evolved populations using a GeneChip Yeast Genome 2.0 Array (Affymetrix). We grew up equal number of cells from the ancestral strain and the evolved populations in YPG medium for 16 h. We then either induced the cells with 0.5 M NaCl or with 1 mM H_2O_2 or grew them uninduced for 20 further minutes as a control. We isolated total RNA using the RiboPure-Yeast RNA isolation Kit (Ambion) and determined the quality of the isolated RNA with a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, DE) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). We only processed samples further if the absorption ratio at 260 nm and 280 nm was between 1.8–2.1 and 28S-18S rDNA ratio was within 1.5-2. We then reverse-transcribed total RNA samples (50 ng) into double-stranded cDNA and then in vitro transcribed the cDNA in the presence of biotin-labeled nucleotides using the GeneChip 3′ IVT Express Kit (Affymetrix). We determined the quality and quantity of the biotinylated cRNA using the NanoDrop ND 1000 and Bioanalyzer 2100. We then fragmented Biotin-labeled cRNA samples (7.5 μg) randomly to 35–200 bp at 94°C in fragmentation buffer (Affymetrix) and mixed in 100 μl of Hybridization Mix (Affymetrix) containing Hybridization Controls and Control Oligonucleotide B2 (Affymetrix Inc., P/N 900454). We then hybridized samples to GeneChip Yeast Genome 2.0 Arrays for 16 h at 45°C. Subsequently, we washed arrays using the Affymetrix Fluidics Station 450 FS450_0003 protocol. We used an Affymetrix GeneChip Scanner 3000 (Affymetrix Inc.) to measure the fluorescent intensity emitted by the labeled target. After hybridization and scanning, we calculated probe cell intensities and summarized for the respective probe sets by means of the MAS5 algorithm (Hubbell et al. 2002). To compare the expression values of the genes from chip to chip, we performed global scaling using a signed-rank call algorithm (Liu et al. 2002). We then transformed the normalized signal intensities logarithmically (base 2). Genes with $\log_2$ transformed values of greater than 4 in all the arrays were considered present in our data.

We carried out microarray analyses 1) for two replicates each for the ancestral strain in YPG, YPGS, and YPGO (six in total); 2) for four replicate population samples for the S populations in YPG, YPGS, and YPGO, that is, 12 analyses in total; 3) for three replicate population samples for the O populations in YPG, YPGS, and YPGO, that is, nine analyses in total; and 4) for three replicate population samples for the SO populations in YPG, YPGS, and YPGO (nine analyses in total).

To identify genes that show physiological expression change in stressor, we considered only those genes for which $|E_{YPGX} - E_{YPG}| \geq 1$ where E_{YPGX} represents the averaged $\log_2$-transformed expression level of a gene in the stressor YPGX over all the replicate measurements and E_{YPG} represents the averaged $\log_2$-transformed expression of the same gene in YPG medium without any stressor averaged over all replicate measurements.

To identify genes whose basal expression or whose regulation changed (see Results), we calculated the average expression levels for the ancestral strain and the evolved populations in YPG, YPGS, and YPGO. For each gene, we then calculated the following two Z-scores.

Z-score for change in regulation

$$Z_r = \frac{(E_{YPGX} - ANC_{YPGX}) - (E_{YPG} - ANC_{YPG})}{\sqrt{0.09 + \sum\left(\frac{\text{Variance}}{\text{No. of replicates}}\right)}}$$

and,

Z-score for basal expression change

$$Z_b = \frac{E_{YPG} - ANC_{YPG}}{\sqrt{0.09 + \sum\left(\frac{\text{Variance}}{\text{No. of replicates}}\right)}},$$

where E_{YPGX} is the average log$_2$-transformed expression level of the focal gene in the evolved populations in YPGX medium (YPGS or YPGO), ANC$_{YPGX}$ is the average log$_2$-transformed expression level of the focal gene in the ancestral strain in YPGX medium, E_{YPG} is the average log$_2$-transformed expression level of the focal gene in the evolved populations in YPG medium, and ANC$_{YPG}$ is the average log$_2$-transformed expression level of the focal gene in the ancestral strain in YPG medium (Mukhopadhyay et al. 2006; Dhar et al. 2011).

The Z-scores take into account the mean expression change of a particular gene, and also the variation in gene expression among the replicates under various conditions (Mukhopadhyay et al. 2006). In both calculations, 0.09 is a pseudovariance (Mukhopadhyay et al. 2006). We calculated the variance term in the denominator of the above equations for the expression level of a gene within replicate arrays in the ancestral strain and within replicate arrays in the evolved populations, always in a given medium. The variance term is divided by the number of replicate arrays and summed over the different media in which the expression values were measured, as indicated by the summation sign in the formula. For calculation of Z_r, we calculated the variances of expression levels for the replicate arrays in each of the following cases: in the ancestral strain in YPG, in the evolved populations in YPG, and in the ancestral strain in YPGS or YPGO and in the evolved populations in YPGS or YPGO. For calculation of Z_b, we calculated the variances of a gene within the replicate arrays in the ancestral strain in YPG and in the evolved populations in YPG. The formulae used here are modified from (Mukhopadhyay et al. 2006) for several reasons. First, in our experiment, we use expression data from three biological replicate populations for each kind of evolved populations (S, O, or SO). Thus, the probability of finding the same genes being differentially expressed in three independent replicate populations by chance alone will be very low. Second, the aim of this analysis was to detect the genes with changed expression that are shared among replicate evolved populations. A gene might not change its expression level to the same extent in parallel evolved populations, thus generating variance in expression among the replicates. To compensate for this increased variance, we set the Z-score threshold for identifying differentially expressed genes lower than the Z-score threshold used in (Mukhopadhyay et al. 2006). Third, we divided the variance by the number of replicates for each condition, as the actual variance would be proportional to the sample variance and inversely proportional to the number of replicates. Overall, we considered genes whose absolute Z-scores exceeded a value of 1.5 to be differentially expressed. We then classified the genes into two main categories of change in regulation, namely change in induction and change in repression, based on the physiological response of these genes to the stressors in the ancestral strain.

The choice of the Z-score threshold determines the minimal change in expression that we can detect, taking the variance among replicates into account. If the sum of the variances in the above equations was equal to zero, and the Z-score threshold was set as 1.5 (as we do here) then all genes with an absolute value of log$_2$(fold change) greater than 0.45 would be identified as genes with evolutionary expression changes. If the variance among replicates is greater than zero, the absolute value of the log$_2$(fold change) would also need to be higher to identify a gene to be differentially expressed. For example, if the sum of the variances among replicates was equal to 1, then the absolute value of log$_2$(fold change) would have to be greater than 1.57 to identify a gene as differentially expressed.

Supplementary Material

Acknowledgments

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