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ARTICLES

Adaptive prediction of environmental changes by microorganisms

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Natural habitats of some microorganisms may fluctuate erratically, whereas others, which are more predictable, offer the opportunity to prepare in advance for the next environmental change. In analogy to classical Pavlovian conditioning, microorganisms may have evolved to anticipate environmental stimuli by adapting to their temporal order of appearance. Here we present evidence for environmental change anticipation in two model microorganisms, *Escherichia coli* and *Saccharomyces cerevisiae*. We show that anticipation is an adaptive trait, because pre-exposure to the stimulus that typically appears early in the ecology improves the organism's fitness when encountered with a second stimulus. Additionally, we observe loss of the conditioned response in *E. coli* strains that were repeatedly exposed in a laboratory evolution experiment only to the first stimulus. Focusing on the molecular level reveals that the natural temporal order of stimuli is embedded in the wiring of the regulatory network—early stimuli pre-induce genes that would be needed for later ones, yet later stimuli only induce genes needed to cope with them. Our work indicates that environmental anticipation is an adaptive trait that was repeatedly selected for during evolution and thus may be ubiquitous in biology.

Microorganisms are constantly faced with environmental stimuli and stresses. The cellular response to such challenges has been intensively studied in several model organisms[1–4]. The simplest response strategy to a stimulus is to monitor the environment and to respond directly to it using designated mechanisms (Fig. 1). The environmental stress response in yeast represents a more complicated strategy in which the responses to many stresses are partially overlapping[1,2]. Theoretical work has shown that when a population of microorganisms evolves under erratic environmental fluctuations, cells may not effectively monitor the environment, but rather use stochasticity to randomly alternate between potential states[5] (Fig. 1). Stochastic switching might thus ensure that a portion of the population is prepared in advance for the unpredicted challenge[6,7]. However, other, more predictable, environments offer organisms the opportunity to adopt an alternative regulation strategy of anticipating an environmental change based on a preceding signal. The capacity of some complex multicellular eukaryotes to capture the statistics that govern the temporal connection between events in their environment, known as classical Pavlovian conditioning, serves as a central paradigm in the study of learning[8]. Here we ask whether genetic regulatory networks of microorganisms adaptively evolved to capture the temporal connections between subsequent stimuli in their environment. Most recently, 'anticipatory regulation' was discovered[9] (Fig. 1)—an association between environmental changes in bacteria. Specifically, this study investigated the response of *E. coli* to temperature increase that is followed by a drop in oxygen availability upon its entry to the digestive tract. Interestingly, these two signals show a symmetrical associative regulation pattern—each signal affects the expression of genes needed to cope with both (Fig. 1). Remarkably, the authors successfully decoupled the two responses during a laboratory evolution experiment in which the two signals were presented out-of-phase from one another. The ability to decouple the two responses is an indication that the coupling seen in the wild type is not a trivial combined response to the two stresses.

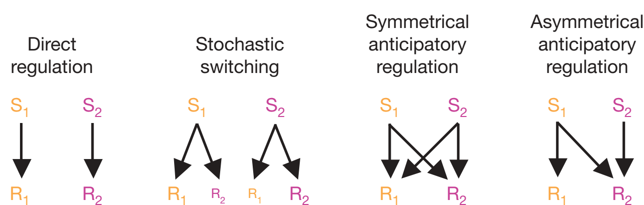

Figure 1 | Four possible regulation strategies in response to environmental stimuli. Under direct regulation, each of the stimuli, S_1 and S_2, activates exclusively the responses R_1 and R_2, respectively. Under stochastic switching, cells randomly sample either R_1 or R_2 in response to either S_1 or S_2. Under symmetrical anticipatory regulation, each of the stimuli activates both responses. Under asymmetrical anticipatory regulation, the stimulus that usually appears first in the ecology activates both responses, whereas the stimulus that appears later induces only the second response.

Here we show that biological systems that react to a unidirectional temporal order of environmental changes may manifest a more elaborate predictive capacity. This capacity is reflected in a corresponding asymmetric response strategy between subsequent stimuli, denoted S_1 and S_2 hereafter, and their designated responses R_1 and R_2, respectively. The first stimulus, S_1, activates both responses, R_1 and R_2, yet because the second stimulus, S_2, does not predict the appearance of S_1, it only activates its own response (Fig. 1). We propose three criteria to determine whether the observed cross-regulation pattern forms an adaptive anticipatory response strategy that could be selected for by evolution. First, asymmetric fitness advantage: pre-exposure to S_1 increases the fitness under S_2, yet pre-exposure to S_2 should not enhance fitness upon subsequent growth on S_1. This ensures that the natural order of stimuli was captured during evolution. Second,

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cost of preparation: pre-induction of genes needed to cope with S_2 is costly and not beneficial during S_1. This indicates that early induction is preserved due to a future benefit that is expected to exceed the cost, upon encounter with S_2. Third, specificity: the conditioned response is specific to S_1 and not to other unrelated stimuli, suggesting that anticipation evolved in response to the specific conditions of the ecological niche.

E. coli carbon source switch

During its life cycle *E. coli* alternates between two principal habitats, intestines of mammals and water, sediment and soil[10] (Supplementary Information). Focusing on the intestinal ecology reveals a predictable metabolic environment. Specifically, during passage along the digestive tract, exposure to lactose precedes exposure to another sugar, maltose[11]. We thus expect that this environment can select for asymmetric anticipation, so that bacteria that link the presence of lactose with future exposure to maltose are expected to activate the maltose genes already upon encounter with lactose. Such pre-induction is expected to allow cells to better use maltose upon its encounter.

We start by examination of anticipatory behaviour at the molecular level. To test systematically the promoter activity of all relevant operons in the presence of maltose and lactose we used an *E. coli* promoter-fused green fluorescent protein (GFP) library previously described[12]. Our experiments revealed the unidirectional regulation pattern expected under a conditioned response strategy (Fig. 2a and Supplementary Figs 1–4). Maltose operons, which are induced by maltose, are also induced, but to a lower level, by lactose. We note that the basic responsiveness of some of the maltose operons to lactose is not restricted only to the *E. coli* strain used in our study[13]. In contrast, and as expected by our hypothesis, the lactose operon shows no response to maltose (Fig. 2a and Supplementary Figs 1–4).

The crucial question is whether the observed asymmetric response strategy that implies anticipation is indeed adaptive—that is, whether it could be selected for during evolution. Following the criteria outlined previously, we first validated that the conditioned response indeed provides an asymmetric fitness advantage in an alternating sugar environment. We monitored cell growth on maltose after an initial growth phase on lactose. Reassuringly, we observed that the wild-type strain displays a fitness advantage if growth on maltose was preceded by growth on lactose (Fig. 3). We ensured that this advantage does not originate from the metabolic contribution of lactose as a carbon source (Supplementary Information). In addition, and as required by our first principle, we found that pre-exposure to maltose before growth on lactose does not improve fitness (Fig. 3).

Our second criterion for adaptiveness requires that pre-induction of the maltose genes is maladaptive or neutral during growth on lactose. This criterion is crucial to rule out the possibility that the maltose genes are induced during exposure to lactose simply because they are needed during that phase. Built into the classical conditioning paradigm from the cognitive context is the possible extinction of the association. To put in present case terms, it is expected that repeated exposure to lactose without consequent arrival of maltose would select for weakening of the conditioned response due to the futile cost of preparation. Accordingly, we have examined laboratory-evolved strains of *E. coli*, which grew for 500 generations on high levels of lactose yet without exposure to maltose[14]. We measured the promoter activity of relevant operons in three lineages that evolved independently under various lactose concentrations. Notably, we found that, in all three lines, the maltose operons show almost no activity in response to lactose (Fig. 2b and Supplementary Fig. 5). Reassuringly, the strains seem to have preserved the ability to activate the maltose genes in response to maltose itself (Supplementary Fig. 6), indicating that only the asymmetrical cross talk between the two pathways was removed during this laboratory evolution period. These results imply that the conditioning observed in the wild-type strain is costly and that without a subsequent benefit that should exceed this cost, purifying selection acts to eliminate this cross talk. Consistent with the second criterion, the growth advantage

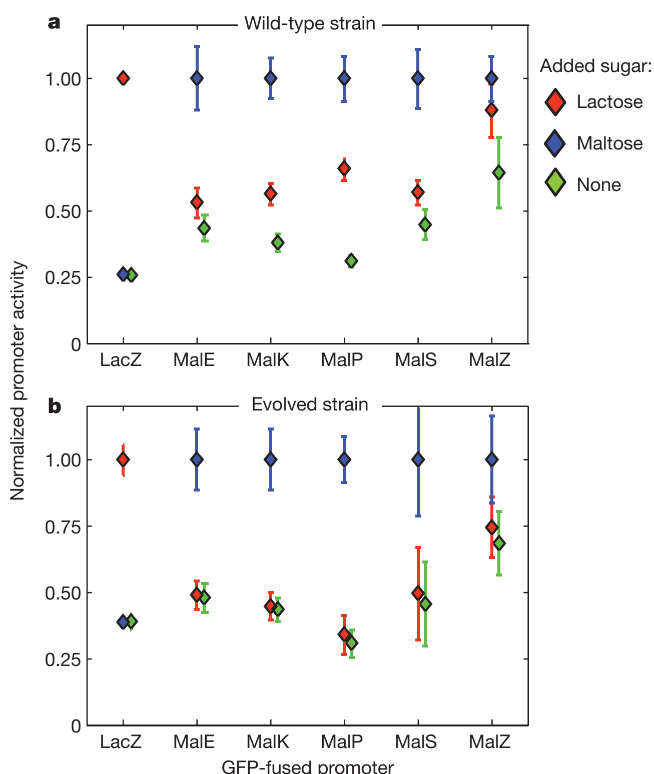

Figure 2 | Conditioned response in *E. coli* sugar metabolism. Promoter activity in wild type (**a**) and the evolved strain (**b**). Colours mark the added sugar. Error bars denote standard deviation of four repeats. Promoter activities of four out of five maltose operons in wild type are significantly higher under lactose relative to the untreated culture (t-test, $P < 0.01$ according to a Bonferroni adjustment). In contrast, none of the promoters show increased activity in the evolved strain. The experiment was done in M9-Glu medium and was repeated using glycerol as a background carbon source (Supplementary Fig. 3) and further verified using quantitative polymerase chain reaction with reverse transcription (RT–PCR) (Supplementary Figs 2 and 4).

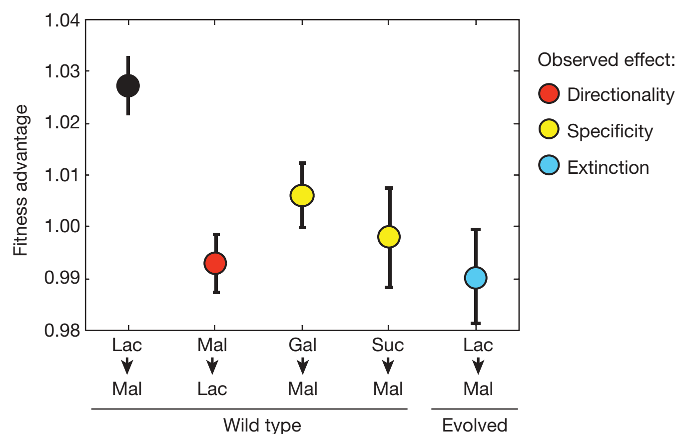

Figure 3 | Fitness in an alternating sugar environment. Two isogenic populations were grown on M9-Gly either supplemented with a sugar as S_1 (treated) or not (untreated). An equal amount of cells was diluted into media containing the second sugar (S_2) as a sole carbon source. The ratio treated/untreated (after 2 h) represents the fitness advantage originating from pre-exposure to S_1. The error bars denote standard deviation of three repeats. All ratios are normalized to the ratio measured in a control experiment in which S_2 was glycerol (Methods). A significant fitness advantage was observed only in the transfer of wild-type cells from lactose to maltose ($P = 0.02$, t-test).

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observed in the wild type due to exposure to lactose before maltose is lost in the evolved strain that features extinction (Fig. 3).

Finally, as required by the specificity criterion, we ensured that pre-exposure to other sugars does not improve growth of cells upon transfer to maltose. As can be seen in Fig. 3, two alternative related carbon sources, galactose and sucrose, cannot substitute lactose as a preceding signal before maltose. This indicates that the observed anticipation evolved in response to the specific stimuli pair that is presented to the organism in its ecological niche. In summary, we conclude that anticipation of subsequent carbon source changes is an adaptive trait that has been selected by the natural ecology of *E. coli*.

The shift from fermentation to respiration in yeast

We next proceeded to a more complicated case that may also manifest environmental change anticipation. We examined how environmental anticipation is encoded in the regulatory network of *S. cerevisiae*, and whether this response strategy has been selected for because of a net fitness advantage it entails. We examined conditions that emulate some of the stresses that occur during the process of alcoholic brew production, such as wine, by yeast[15]: Fig. 4a describes this process, which starts at a highly osmotic and low pH environment, followed by potentially lethal temperature raise due to vigorous fermentation and accumulation of ethanol, culminating in the diauxic shift—the exhaustion of fermentable sugars (for example, glucose) and the switch to oxidative respiration[16–19]. Respiration is challenging to the cell because it eventually generates oxygen radicals[20]. We checked whether yeast cells can cope better with these stresses provided that they appear in their natural temporal order, that is, whether yeasts use early stresses as predictive signals for the likely arrival of later stresses. Such behaviour would indicate that yeast cells are preparing for later challenges even before they arise.

To address these questions we systematically studied the survival of *S. cerevisiae* under stresses that comprise the switch from its preferred catabolic state, fermentation, to the alternative one, respiration (Fig. 4). As a potential manifestation of anticipation, we focused on the cross-protection phenotype between different stress pairs[21,22]. A cross-protection phenotype between two stresses exists if pre-exposure to one stress improves the survival of cells under a subsequent stress. Under our hypothesis we expect an asymmetrical cross-protection phenotype between stress pairs. Particularly, we predict that directionality of cross-protection will be in accordance with the order of events during the transition from fermentation to respiration. For example, because heat shock and ethanol accumulation precede oxidative stress in the ecology, we expect improved survival under oxidative stress if it is preceded by either heat shock or ethanol stress, yet we do not expect cross-protection if the stresses are applied in the reverse order. Figure 4a shows the measured protection phenotype systematically tested for stresses that occur before and after the diauxic shift. In accordance with our hypothesis, we observe that the cross-protection phenotype is indeed extremely asymmetric and, most importantly, that the directionality of protection fits the natural temporal context. Specifically, for each stress pair protection is always stronger in the direction found in the natural environment, thus fulfilling the asymmetric fitness advantage criterion. In a more faithful emulation of the natural habitat we have also applied the three stresses, heat shock, ethanol and oxidative stress, sequentially and observed an equally high cross-protection activity (Supplementary Information). Note that activation of the

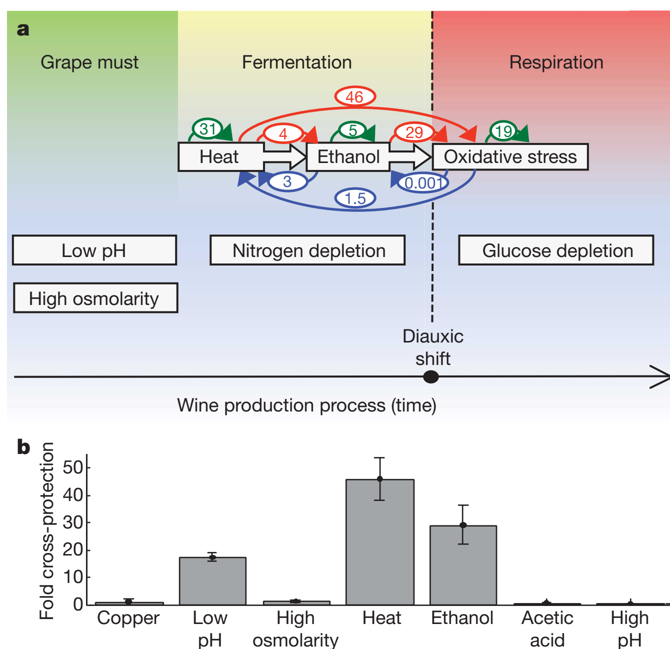

Figure 4 | Cross-protection in the context of the diauxic shift. a, Stress sequence in the process of wine production and measured cross-protection phenotype. 'Must' is the medium in the beginning of the wine production process. The values denote the fold protection gained by pre-exposure to a mild stress followed by a severe stress. Red and blue arrows mark cross-protection when stress order is in accordance with the ecology or in reverse order, respectively. Green arrows denote auto-protection. **b**, Specificity of cross-protection against oxidative stress. The columns mark the observed fold cross-protection provided by exposure to mild stress before severe oxidative stress. Error bars donate standard deviations of three repeats.

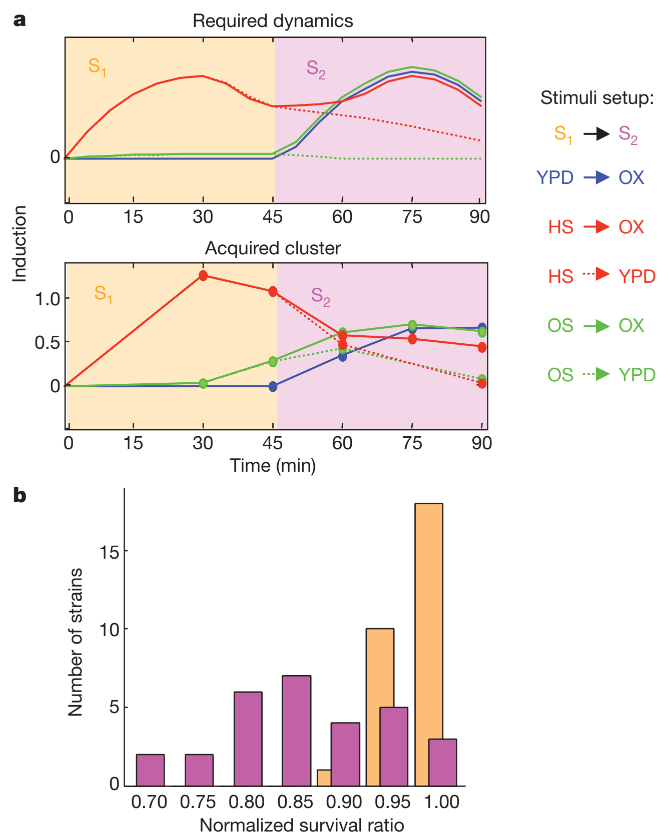

Figure 5 | Candidate genes underlying the asymmetrical protection between heat and oxidative stresses. a, Genome-wide expression was measured in response to sequential stress pairs (untreated, YPD; osmotic stress, OS; heat shock, HS; oxidative stress, OX). The top panel marks the expression dynamics of an ideal gene that may facilitate cross-protection against oxidative stress. The bottom panel marks the mean profile of 300 genes matching the desired pattern (Methods). **b**, A histogram of sensitivity values of 29 deletion strains to heat (orange) and oxidative (purple) stresses. The survival of each strain was normalized to the survival of the wild type to test for increased sensitivity (individual strain sensitivity is shown in Supplementary Fig. 7 and Supplementary Table 5).

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environmental stress response[1,2], namely a set of genes that are transcribed in response to a wide spectrum of stresses, is less likely to explain the observed cross-protection activity. Whereas the environmental stress response is more likely to result in a symmetrical protection, we observe a very asymmetric effect. Furthermore, and as required by the third criterion, we observe that protection against oxidative stress is specific to stresses that precede respiration in the natural environment, whereas stresses unrelated to the process do not provide cross-protection (Fig. 4b).

We next investigated a potential molecular mechanism that might account for the observed cross-protection capacity. We focused on the pair of stresses that exhibits the strongest cross-protection phenotype, heat shock and oxidative stress, and measured genome-wide gene expression in response to these stresses when they were introduced in isolation or one after the other. In particular, we applied a heat shock as the first stress (S_1), followed by an oxidative stress (S_2). As a control we also examined the response to oxidative stress that is preceded by an osmotic stress, a condition we found not to cross-protect significantly against oxidative stress (Fig. 4b). We used these series of events to define an ideal expression profile of genes that can underlie the cross-protection phenotype and ultimately the anticipation capacity (Fig. 5a, see Methods for profile details). We also defined a control profile, using the control osmotic stress (instead of heat shock) as an early stimulus. Our search revealed a cluster of 300 genes with the desired profile (Fig. 5a). Consistent with asymmetric anticipation, this cluster shows a significant overlap with a set of genes that are annotated in the literature (Proteome database) as essential in oxidative stress but not in heat shock ($P = 0.013$). However, this cluster does not overlap significantly with a set of genes that are annotated as essential in heat shock and not in oxidative stress ($P = 0.2$). Analysis of functional enrichment using Gene Ontology annotations[23] revealed a similar trend—a significant enrichment of oxidative stress categories (Supplementary Table 3), such as oxidoreductase activity, and many mitochondria-related categories, consistent with a known role of mitochondria for coping with oxidative stress in yeasts[24]. However, no heat-shock-related categories were found to be enriched in this cluster. Reassuringly, the genes that corresponded to the control profile (in which we still used oxidative stress as an S_2, yet with osmotic stress as an unprotective S_1) did not yield similar enrichment for oxidative-related activities (Supplementary Table 4). This indicates that whereas an S_1 stimulus that provides cross-protection increases in advance the expression level of genes relevant for the R_2 response, a control S_1 signal does not affect these R_2 genes.

We then tested whether induction of the conditioned gene set is neutral in heat shock, as required by the second criterion. Towards this aim we collected 29 strains, each of which is deleted for one of the genes from the cluster described above, and tested their sensitivity to heat shock and oxidative stress (Methods). Analysis of survival ratios reveals that whereas 65% of the strains show increased sensitivity to oxidative stress, none shows a significantly increased sensitivity to heat shock relative to the wild-type strain (Fig. 5b and Supplementary Fig. 7). This is a direct indication that the expression of many of the heat-induced genes is at least neutral, if not maladaptive, under heat shock. Taken together with the fact that these genes are essential under oxidative stress, this experiment corroborates the claim that an early preparation to oxidative stress during heat shock is adaptive in this system.

Future outlook

An open question is how prevalent anticipatory regulation is in the world of microorganisms. One way to address this issue is to develop a mathematical framework that estimates the potential advantage of a conditioned response in a given ecology. Intuitively, the added value from anticipation depends both on environmental and on biological parameters. Two cellular parameters, gain and cost, should be considered. A conditioned response is beneficial provided that benefit gained from anticipation exceeds the cost of early preparation. These parameters are in turn dependent on the typical time constants of the environment, for example, the time gap between the appearances of the two stimuli. Additionally, predictable environments in which the stimuli are often sequentially coupled promote anticipation. A simple equation captures the relationship between these variables and their effect on the net fitness:

$$\Delta F = p(B(\Delta t) - C(\Delta t)) - (1 - p)(C(t_{S1})),$$

where ΔF is the difference in fitness between an anticipating cell and a cell that adheres to direct regulation (Fig. 1), p is the probability that S_2 will occur given that S_1 occurred, and B and C are functions describing the benefit and cost of early preparation, respectively. Δt is the length of the time interval between the appearance of S_1 and S_2, and t_{S1} is the duration of the first stimulus. The dependence of fitness on the interplay between the key factors is shown in Supplementary Fig. 8. We note that quantitative predictions from the above phenomenological equation can be drawn by incorporating into the model biologically meaningful and realistic parameters, such as measurable gain, cost and timescales (A.M. *et al.* in preparation).

To conclude, as we observed that anticipation at the cellular level appears to be adaptive both in prokaryotes (*E. coli*) and eukaryotes (*S. cerevisiae*), and under remote systems such as sugar metabolism and stress response, we propose that it may be prevalent in a diverse range of species. Asymmetric anticipation, along with associative learning[9], memory[25] and physiological adaptation[26], represent a powerful set of tools used by microorganisms to achieve a sophisticated environmental response.

METHODS SUMMARY

Strains. *E. coli* MG1655 was used for most experiments. An *E. coli* GFP reporter library[12] was used to monitor operon expression. The evolved *E. coli* strains were described previously[14]

S. cerevisiae BY4741 (*MAT**a***; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) was used in all yeast experiments. All deletion strains were obtained from the *Saccharomyces* Genome Deletion Project[27].

***S. cerevisiae* cross-protection.** An overnight culture was diluted into fresh YPD medium and grown to a concentration of 2×10^6 cells per ml. Cells were diluted 1:2 into the first stress (S_1). Transfer into the second stress (S_2) was done by adding treated medium after centrifugation. Stresses were calibrated to achieve a mild effect (~50% survival) for S_1 and a severe effect (~0.5% survival) for S_2 (Supplementary Table 1). Samples were taken from cultures at the end of each treatment and plated. Each experiment was carried out in three repeats. The fold protection was calculated as described in Supplementary Equation (1).

***E. coli* fitness advantage.** Overnight cultures were diluted into M9-Gly plus S_1 (for example, lactose; treated) or M9-Gly (untreated). After 3 h, population size was determined and used to dilute (~1:100) an equal amount of cells into a new growth medium containing low levels of S_2 (for example, maltose) as a sole carbon source. To account for a potential metabolic effect of residual S_1, an identical trace amount of S_1 was added to the untreated growth medium. The ratio treated/untreated after 2 h of growth was used as an indication of fitness advantage originating from pre-exposure to S_1. To rule out the possibility that the growth advantage observed due to early exposure to lactose originates from its metabolic value rather than its rule as a conditioning signal, we preformed a control experiment (Supplementary Information).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.M. raised the original idea and performed all the experiments; G.R., B.G. and A.Y. participated in experiments; E.D. evolved the *E. coli* strain; A.M., O.D. and Y.P. designed the experiments; A.M., M.K., O.D. and Y.P. analysed the data; O.D. and Y.P. supervised the project; A.M., O.D. and Y.P. interpreted the results and wrote the manuscript.

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METHODS

***S. cerevisiae* media.** All experiments were carried out in YPD medium (2% yeast extract, 1% peptone, 1% dextrose) at 30 °C.

Sensitivity of deleted *S. cerevisiae* strains to heat and oxidative stress. To examine the importance of the conditioned gene cluster under heat and oxidative stresses, 29 genes were chosen for additional experiments. This subset of genes exhibited a two-phase induction profile: initial induction under heat shock that further increased under oxidative stress. We then used strains in which each of the 29 genes was deleted to check for increased stress sensitivity as follows.

Cultures were grown to stationary phase in a 96-well plate, diluted (1:20) into fresh YPD medium and grown for additional 3 h. Cells were then diluted into either mild heat shock (37 °C for 30 min) or mild oxidative stress (H_2O_2 1 mM for 30 min). The stresses were calibrated to achieve 90% survival in the wild-type strain. As a control, untreated cultures were diluted into YPD. All cultures were then diluted (1:20) into fresh YPD and grown for 6 h. Population size was monitored using a multi-well spectrophotometer at 595 nm. Survival ratio was calculated by dividing the optical density of stress-treated cultures by the optical density of untreated cultures. A *t*-test was used to determine whether the difference in the survival ratio of a deletion strain and the wild type is statistically significant in a given stress.

Genome-wide expression experiments. An overnight culture was diluted into fresh YPD and grown to concentration of 2.5×10^7 cells per ml. Cells were diluted 3:4 into a fresh medium containing S_1 (KCl 0.8 M, heat shock 40 °C or YPD) and grown for 45 min. Cells were then diluted 1:10 into S_2 (H_2O_2 0.66 mM or YPD). Stress levels were calibrated to have relatively minor effects on survival. Aliquots were removed 0, 30 and 45 min after the addition of S_1 whereas in S_2 aliquots were removed after 15, 30 and 45 min. RNA was extracted using MasterPure, followed by hybridization to Affymetrix yeast 2.0 microarrays. The expression data set was deposited in the GEO database (GSE15936).

Identifying candidate genes that facilitate conditioning. We defined an ideal expression profile of a gene that can facilitate the observed cross-protection phenotype (heat shock but not osmotic stress protects against oxidative stress). The profile is defined accordingly: (1) induction in response to heat shock is 0.2 or higher in $\log_2$ scale; (2) induction in response to oxidative stress is 0.2 or higher in $\log_2$ scale; (3) induction under osmotic stress is significantly lower than in heat shock, 0.4 in $\log_2$ scale; (4) induction is maintained higher in the transfer from heat shock to oxidative stress relative to transferring from heat shock to YPD.

A control profile is similarly defined by using osmotic stress instead of heat shock in the above requirements. The results are qualitatively similar under alternative cut-off values (not shown).

***E. coli* media.** All experiments were done in M9 medium (1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.05% casamino acids and 5 ng ml^{-1} thiamine), supplemented with the appropriate carbon source.

Three basic media were used: M9, M9-Gly (0.1% glycerol) and M9-Glu (0.1% glucose plus 20 mM cAMP; cAMP was added to avoid glucose repression[28]).

To test the effect of various treatments the media were supplemented with the following: 10 mM lactose, 5 mM maltose (these concentrations allow maximal growth), 10 mM galactose, 10 mM sucrose (these concentrations are equal to the concentration of lactose), 0.15 mM IPTG (this concentration achieves saturation of the lactose operon induction[29]) and 25 μg ml^{-1} kanamycin.

Monitoring operon transcription using the promtor-fused GFP library. Overnight cultures, each carrying a unique plasmid with a specific promoter fused to GFP, were diluted into fresh M9-Glu media, grown for 1 h at 37 °C and then treated with sugars. Expression and cell growth (at 30 °C) were monitored simultaneously using a multi-well reader (fluorescence at 495/520 nm, optical density at 595 nm). The normalized GFP level and promoter activity were calculated similarly to in ref. 28. Briefly, the GFP signal was calculated after subtraction of the medium fluorescence and cell autofluorescence. GFP per cell was calculated dividing the GFP by the optical density. Promoter activity ((dGFP/dt)/optical density) was taken as the average promoter activity measured in a time window of 1 h of exponential growth.

The following operons were examined in our research: MalEFG, MalK-lamB-malM, MalPQ, MalS, MalZ, LacZYA (referred to by the name of the first gene). MalT was not included in our analysis because it shows no responsiveness to maltose.

Monitoring operon transcription using the quantitative RT–PCR. Overnight cultures were diluted into either M9-Gly or M9-Glu, alone or supplemented with either lactose or maltose, and grown until the logarithmic phase was reached. RNA was extracted using RNeasy Mini kit and used as a template for quantitative RT–PCR (LightCycler 480 system).

28. Kaplan, S. *et al.* Diverse two-dimensional input functions control bacterial sugar genes. *Mol. Cell* **29**, 786–792 (2008).
29. Dekel, E. & Alon, U. Optimality and evolutionary tuning of the expression level of a protein. *Nature* **436**, 588–592 (2005).

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