Programmed bet hedging in yeast responses to osmotic stress

Yoshikazu Hirate[1], Samuel Bottani[2], Wyming Lee Pang[3] and Suzannah Rutherford[1]

1 Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, 2 Laboratoire Matière et Systèmes Complex, Paris Diderot, Paris, France 3 Department of Bioengineering, University of California San Diego, San Diego, CA

Abstract

Rapid reproduction contributes to evolutionary fitness but can be lethal under stress. Microbes are thought to limit risk in clonal populations by bet hedging; the stochastic expression of a low frequency of slow growing cells constitutively resistant to unpredictable environmental stresses including antibiotics. However fitness depends on rapid recovery and resumption of growth in potentially lethal environments whose severity and duration are also unpredictable. Here we describe trade-offs between osmotic stress-responsive signaling, survival and proliferation in 50 ecologically distinct strains of budding yeast. By contrast with prior examples, programmed bet hedging responses were heritable, stress-specific and varied continuously in our population. During rapid growth strong osmotic stress signaling promoted survival. Weak signaling predicted lower viability, intense rebound signaling and robust recovery. Older cultures survived and adapted to unprecedented stress with fitness depending on reproducible, strain-specific proportions of cells with divergent strategies. The most 'cautious' cells survive extreme stress without dividing; the most 'reckless' cells attempt to divide too soon and fail, killing both mother and daughter. Heritable proportions of cautious and reckless cells generate a tunable, rapidly diversifying template for microbial bet hedging that resembles natural variation and would evolve in different patterns of environmental stress.

In order to understand the evolutionary trajectories of populations and species we need to understand the effects of natural genetic variation on mechanisms of development and expression of phenotypic variation. The mapping between genetic variation and the spectrum of attributes and behaviors upon which selection acts defines population-level properties such as evolvability (the capacity to evolve), robustness or canalization (the capacity to withstand genetic and environmental perturbation), and reaction norms (the optimization, within a given genotype, of phenotypic responses across different environments)[1–3]. Here we describe strain-specific differences in hyperosmotic stress responsive signaling and associated behaviors in a synthetic population of budding yeast.

The high osmolarity glycerol (HOG) signaling pathway is central to an elaborate stress response that reduces cellular damage and death in unpredictably changing osmotic environments where the balance between external solutes and free water pressure in the cell can change suddenly[4]. A main function of the HOG pathway is the production and accumulation of intracellular glycerol, which restores water balance and, as demonstrated by a large body of work from many labs, is essential for survival, adaptation and proliferation in hyperosmotic stress[4–9]. In the wild, yeast and other microorganisms must balance immediate survival against evolutionary fitness. Multiplicative fitness favors clonal populations that respond as rapidly as possible to improved conditions with earlier cell cycle reentry and proliferation[10]. On the other hand, individual survival requires that cells carefully sense the amplitude and direction of environmental change to more safely reenter the cell cycle after stress[7]. The HOG pathway consists of at least two highly-conserved, multi-component osmotic stress sensors linked to a parallel series of at least 15 kinases and accessory proteins that ultimately alter the activity of nearly 10% of the yeast genome[4,6]. The sheer numbers of genes involved in HOG signaling, their conservation, and their elaborate circuitry suggest that a nuanced response to osmotic stress has been crucial and strongly selected throughout evolutionary history. As the hyperosmotic stress response of budding yeast has well-characterized and accessible signaling and phenotypic traits that can be measured in the lab and are almost certainly under strong selection in nature[4,6,7], this system is ideal for characterizing the mapping between signaling and behavior in a diverse population.

We measured osmotic stress signaling, survival and adaptation in both exponentially growing and nearly quiescent cultures of diploid yeast. Glycerol-3-phosphate dehydrogenase (*GPD1*) is rate-limiting for glycerol production[11] so we used the synthesis and accumulation of green fluorescent protein (GFP) integrated into the gene for *GPD1* as a proxy for HOG pathway activity. A synthetic population of diverse yeast genotypes was made by crossing *GPD1*::GFP in the genetic background of a standard laboratory strain (BY4742 *MATalpha*) to a panel of wild and industrial genetic backgrounds –e.g. fifty different haploids of the opposite mating type extracted from globally diverse, sequence-validated strains of *Saccharomyces cerivisiae* deposited to the collection of the Royal Netherlands Academy of Arts and Sciences over the past 100 years (CBS; Table 1 and Table S1).

Osmotic stress signaling and behavior in young cultures

The behavior of single cells before and after their exposure to osmotic stress was followed by time-lapse video microscopy of monolayer cultures in custom microfluidics devices[12]. When cells in exponential growth were exposed to sudden hyperosmotic stress, cell volume decreased, cell division and budding immediately stopped, and daughter cells retracted[13]. After a lag period proportional to the severity of the stress GFP fluorescence driven by the *GPD1* promoter began to accumulate in the cytoplasm of surviving cells. Cells that did not accumulate *GPD1*::GFP to high levels did not survive or adapt, developed large vacuoles, and began to die, remaining in view as shrunken cell ghosts. As GFP accumulated to saturation levels in the surviving cells, they adapted to the higher osmotic pressure, resumed cell division, budded and began to divide with a longer half time, producing daughter cells with similarly high fluorescence[13].

Viability per culture and *GPD1*::GFP accumulation per cell were measured using flow cytometry of statistically large numbers of cells from all 50 strains (~10,000 cells / sample). The rate and extent of mean *GPD1*::GFP accumulation in exponentially growing cultures exposed to hyperosmotic media depended on the severity of the stress and the genetic background of each strain (Figures 1A and S1). Prior to the osmotic stress mean *GPD1*::GFP fluorescence and viability were uncorrelated. After 2 hours in moderate 0.75 M KCl viability decreased and became steeply correlated with accumulated *GPD1*::GFP (Figures1B and C). As expected, natural variation in the strength of HOG signaling was directly responsible for variation among the strains in osmotic stress survival.

Negative feedback drives a robust recovery

The initially strong positive correlation between variation in *GPD1*::GFP accumulation and variation in viability reversed as cells adapted and began to divide (Figure 1C; 4 hours). This distinguished two phases of the response, an early phase (0 – 2 hours) when viability decreased markedly and acute HOG signaling promoted osmotic stress survival and a later phase (2 – 4 hours) when viability recovered but became negatively correlated with HOG signaling and *GPD1*::GFP accumulation. The switch from positive to negative correlations might have indicated that stronger HOG signaling, initially beneficial, suddenly caused lower viability. However we think it likely that negative feedback increased signaling in the surviving cells of the less viable strains. Negative feedback controls, occurring at many levels and timescales, are present in essentially all of the varied mechanisms that act in concert to increase intracellular glycerol and restore water balance. For example (1) unequal water pressures activate osmotic stress sensors, glycerol channels and other pressure-sensitive components whose activities control and depend on water balance (e.g. see Figure 5 in Hohman 2002[4,6]), (2) GPD1 indirectly controls and is controlled by osmotic stress-sensitive kinases that respond to upward and downward changes in water balance[14], and (3) nuclear Hog-1 MAP kinase increases the transcription of phosphatases that restore its own cytoplasmic localization and basal activity[15–17].

Consistent with acting negative regulation, we found there was a strong and highly significant correlation between early mortality (0 – 2 hour decreases in viability) and later accumulations of *GPD1*::GFP (2 – 4 hours; Table 2). We reasoned that cells and strains that adapt quickly experience lower and less sustained effects of osmotic stress (e.g. water loss) with more rapidly attenuated HOG pathway activity and lower *GPD1*::GFP accumulation. Conversely, surviving cells of strains that were slower to adapt and less viable would experience higher and more sustained osmotic stress (and likely other stresses). Prolonged osmotic stress would sustain HOG signaling and maintain *GPD1* transcription – also activated by general stress responses[18] – further promoting *GPD1*::GFP accumulation (e.g. negative feedback regulation of viability at the level of general stress responses). Indeed, even as *GPD1*::GFP and viability became negatively correlated, their *rates of change* remained positively correlated (Figure 1C, 2 – 4 hours and insets; Table 2) prompting a parsimonious interpretation that osmotic stress signaling promotes adaptation and viability during both the initial and recovery phases of the response.

By 4 hours all strains had adapted to a new steady state in 0.75M KCl and later viability remained largely unchanged (Figure 1C inset, lower right). Interestingly, initial decreases in steady-state viability (0 – 2 hour mortality) were almost perfectly restored by 4 hours (Figure 1D) and, remarkably, by 6 hours early mortality and recovery were over 98% correlated (R^2 = 0.9852, P<0.0001; not shown). The biological robustness of adaptation and complete recovery of steady state viability further support the idea that negative feedback restores viability through continued activation of stress responses. Indeed, the continued accumulation of *GPD1* and glycerol – directly responsible for restoration of water balance and reduction of osmotic stress – suggests that intracellular glycerol concentrations integrate the cumulative activities of many facets of the osmotic stress response (e.g. provides a plausible biological mechanism for "integral feedback" that virtually assures perfect adaptation[17,19]; see included manuscript in preparation). However, despite their resilience, strains that were relatively slower to adapt would be ultimately less fit than rapidly adapting strains due to their higher death rate, slower recovery, and lower viabilities before and after adaptation.

Extreme stress resistance of older cultures

By contrast with exponential cultures, when the aging yeast cultures (post-diauxic) were exposed to hyperosmotic media they survived and adapted after long periods in unprecedented conditions (Movies 1 and 2). As aging cultures deplete available glucose in their media they undergo a metabolic change called the diauxic shift[20]. During post-diauxic growth stress response proteins accumulate, cell division slows and then stops, and cells enter quiescence[21]. Remarkably, post-diauxic cultures survived up to 5 weeks in 3 M KCl (41/50 strains). They could not adapt and did not grow in 3 M KCl, but recovered rapidly and grew when plated on fresh isotonic media ('static viability'; Figure S2). When we tested their limits of adaptation in increasing concentrations of KCl all but one strain could grow on 2.6 M KCl media and three strains could

grow on media containing 2.9 M KCl (Table 3). We are unaware of previous reports of such extreme osmotic stress survival or adaption limits for budding yeast of any growth stage or genotype.

Heterogeneity of cells in older cultures

By contrast with cultures in exponential growth, in post-diauxic growth the genetically identical cells within each strain and culture were surprisingly heterogeneous in their signaling behaviors (compare Figures S1 and S3). Neither total *GPD1*::GFP fluorescence nor rates of change in fluorescence was strongly correlated with viability. After several hours in 2.5 M KCl *GPD1*::GFP increased sharply in one group of cells as they began to divide. More surprising, other cells induced *GPD1*::GFP to high levels, started to divide and then popped, killing both the mother and daughter (Movie 3). Another group of cells had slower signaling and cell division while the most 'cautious' groups of cells failed to signal or divide but remained in a cellular state of static viability without dividing.

We us flow cytometry to follow *GPD1*::GFP accumulation and survival of post diauxic cultures exposed to 2.5 M KCl as their osmotic stress responses evolved over 7 days. A Gaussian mixture model with Bayesian information criteria was used to assign the cells in each sample to four Gaussian distributions (G_0-G_3) described by eight parameters – means and covariances – numbered according to their increasing levels of fluorescence (Figure S3 and (https://figshare.com/s/8147275b62eb8d4db6bf/)). Only *GPD1*::GFP pre-accumulated into cells of the G_3 distribution during post-diauxic growth and *prior* to the osmotic challenge –therefore unrelated to osmotic stress signaling – predicted survival at any time. The amount of *GPD1*::GFP accumulated in G_3 cells at time 0 predicted each strains early but not later viability, and this relationship was better fit by 2nd order quadratic rather than linear functions of *GPD1*::GFP (Table 4), suggesting early survival was higher in strains with intermediate G_3 accumulations (more variation explained and lower mean square errors). Despite the fine-scaled characterization of osmotic stress signaling behaviors of the different groups of cells in each strain, none of the distributions learned by the Gaussian mixture model, neither pre-accumulated G_3, total *GPD1*::GFP fluorescence, nor stress-induced *GPD1*::GFP in any distribution, embodied features of osmotic stress signaling important for later survival.

Continuous variation in stress responses

In order to map osmotic stress-responsive signaling onto survival more directly we next quantified osmotic stress signaling behaviors of cells as they unfolded over time in 2.5 M KCl directly, unbiased by Gaussian assumptions or approximations. In brief, normalized levels of induced *GPD1*::GFP were binned (in a histogram or on a grid), creating a 'sample vector' of cell numbers in each of 100 successive intervals of fluorescence intensity (see methods). Next, sample vectors for increasing time points in each strain were linked to create time line vectors of sequential. For comparison of averaged strain behaviors the time line vectors were clustered

using a mean distance matrix, constructed from 17,000 randomly chosen permutations of the data over replicate samples and times (mean clusters; see methods). Finally, the fraction of permutations in which each strain grouped with more than half of the other strains in its mean cluster was used to rank that strain's behavior relative to the other strains in its group (clustering statistics; Table S2).

The unique signaling trajectories of most strains were highly reproducible (Figure S3 and (https://figshare.com/s/8147275b62eb8d4db6bf/)). Based on their shared and strain-specific (heritable) signaling behaviors the 50 strains rapidly converged onto two large groups made up of six mean clusters (Figure 2). Each strain could be further ordered within and between mean clusters based on their clustering statistics, with their rank order describing increasingly rapid accumulations of *GPD1*::GFP and 'reckless' signaling (Figure S4). Tellingly, both mean cluster and rank predicted viability over time (Figure S5 and Table 4; respectively) thereby confirming the biological relevance of 'cautious' versus 'reckless' osmotic stress signaling, validating our clustering method and supporting the role of natural osmotic stress signaling differences between strains in shaping variation in fitness during osmotic stress.

Evidence for bet hedging

As cautious and reckless behaviors were found both within and between strains, we wondered whether bet hedging, the expression of alternate, conditionally-adaptive phenotypes within a clone of genetically identical organisms[10,22–25], could explain the observed variation in osmotic stress signaling and survival. For example, a low frequency of post-diauxic cells that are stress-resistant under normal growth conditions could represent the stochastic, pre-adaptive bet hedging previously documented in yeast and bacteria[26,27]. By contrast with previously described microorganism bet hedging, the ranked responses to osmotic stress responses were heritable, stress-specific and uncorrelated with pre-adapted osmotic stress resistance acquired during post-diauxic growth. The viability of different strains varied depending on their rank and the severity of the osmotic environment. Higher-ranked strains with more aggressive osmotic stress signaling strategies favored milder conditions, but with increasing time in extreme osmotic stress more cautious strains and behaviors became more fit (Table 4). For example, W178 at rank 50 was most viable in moderate 0.75 M KCl, but the optimum shifted to the strain at rank 25 after 20 hours in 2.5 M KCl, 20 after 72 hours, and 9.6 after 168 hours (1 week). After 168 hours, viability had decreased most among the most reckless strains (Figure S5).

The correlation between relative fitness and signaling behavior under increasing osmotic stress provided strong empirical evidence for bet hedging[23]. To confirm that the increasing survival of strains with cautious signaling behaviors was correlated with the amount of osmotic stress experienced by the cells and not simply their time in culture, we tested the idea that the severity of osmotic stress increases with time in severe osmotic conditions. We again incubated cultures in 2.5 M KCl for 168 hours, but they were first exposed to a mild pre-stress (2 hours in 0.5 M KCl) to pre-induce osmotic stress proteins and make them more resistant to

subsequent stress. If optimum rank depended solely on time, independent of the degree of stress experienced by the different strains, then it should be unaffected by the short pre-stress. However optimum rank shifted toward more reckless behaviors (rank 9.6 to rank 18; $P<0.0001$) in response to the pre-stress and viability increased by ~10%, as expected if the pre-treated cells experienced lower osmotic stress.

After 168 hours in 2.5 M KCl the most reckless cells in the highest-ranking strains began to selectively die and disappear. For example, the replicate cultures of strain W242 (rank 49) that had lower viability also had fewer cells with high accumulations of *GPD1*::GFP, smaller G3 distributions, and correspondingly larger distributions with lower mean *GPD1*::GFP (Figure 3A). The selective loss of cells with the highest accumulations of *GPD1*::GFP could indicate that *GPD1*::GFP levels simply decrease over time. However, G3 distributions were stable over most time points and in most strains. We think it more likely that after 168 hours in 2.5 M KCl the most aggressive cells in the highest-ranking strains attempt to divide and fail (e.g. Movie 3). Rapid signaling and adaptation, a fitness advantage in milder conditions, becomes a liability in severe or prolonged osmotic stress. On the other hand static viability – the survival of non-dividing cells in 3 M KCl (Figures 2B and S2) – would usually dramatically reduce evolutionary fitness but it allows more cautious cells and strains to survive severe stress.

Evolution of bet hedging

We've shown that heritable osmotic stress signaling behavior predicts survival in increasing severity and duration of osmotic stress. Since cautious and reckless strains reliably express a range of cells with different behaviors and fitness depending on the environment, we wondered whether a simple, 2-state bet hedging model with heritable proportions of cautious and reckless cell types could account for the observed variation in osmotic stress signaling and explain the complex relationship between rank and viability. We reasoned that aggressive osmotic stress signaling with rapid recovery and resumption of growth would have been the default, ancestral behavior and asked whether a heritable probability of cautious signaling and behavior could have arisen in response to the unpredictable severity and duration of potentially lethal osmotic environments. In short, we assumed a heritable probability of daughters with cautious signaling and behavior (P) and asked whether it could evolve.

We modeled the relative fitness of strains with different strategies P ($0 \leq P \leq 1$) after several generations of growth under abrupt changes between three very general osmotic stress environments (https://figshare.com/s/2c03544aef0c40cc86c2/). These environments discriminate cautious versus reckless behaviors: (E0) a permissive environment in which both cautious and reckless cells grow equally well, (E1) a restrictive environment approximating moderate osmotic stress reckless cells divide and cautious cells survive without dividing, and (E2) a killing osmotic stress where reckless cells die and cautious cells survive without cell division. After several generations under each of the 9 possible environmental shifts between the three environments, we calculated the relative fitness (cell numbers) of each strategy. Most environmental shifts

favored an optimum strategy of either all cautious (P = 1) or all reckless cell types (P = 0; Figure 3C). Strictly intermediate strategies (0 < P < 1) and bet hedging prevailed only when the osmotic environment shifted from moderate to more severe, with the optimum P depending on the number of generations in the first environment (E1 -> E2). Shorter lag periods – corresponding to less severe osmotic conditions – and more cell divisions in E1 initially favor lower P and a higher proportion of reckless cells. Longer lag periods – corresponding to more severe conditions and fewer cell divisions – favor higher P and a higher proportion of cautious cells. Indeed, worsening osmotic environments are common in nature (for example, during fermentation or drying). The post-diauxic cells and strains in our experiments experienced a worsening environment with increasing time in osmotic stress; as predicted by the model, lower-ranked strains with more cautious signaling behaviors, longer lag periods and fewer attempted cell divisions were increasingly fit over time and with an increasing severity of osmotic stress (Table 4).

While a 2-state model of bet hedging behaviors in three discrete environments is almost certainly overly simplistic relative to yeast in nature (and up to four learned distributions of cell types that best fit our data; Figure S3), it provides a conceptual framework for understanding variation in osmotic stress signaling strategies and generates testable hypotheses for further studies. Bet hedging in microorganisms has been previously thought to arise almost exclusively through stochastic switching with a low probability of alternate phenotypes that are independent of the environmental challenge[10,26–28]. By contrast, the variation in osmotic stress signaling and behavior we report here is a heritable, programmed response to osmotic stress. Strains display a wide range of cells displaying cautious behavior making this example one of few known for a microorganism that is similar to the rapidly diversifying, within-clutch bet hedging strategies of plants and animals[10,28]. If the heritable probabilities of cautious cells and behavior were sculpted by selection, milder and/or slowly worsening osmotic environments would generate more reckless strains (e.g. W242, rank 49 isolated from rotting banana; W282, rank 48 isolated from fermenting fruit) and more severe or rapidly deteriorating osmotic environments would generate more cautious strains and behavior (e.g. W455, rank 1 from molasses; W217, rank 4 from sugar cane syrup). Indeed, even though our synthetic population is represented heterozygous wild/lab diploids rather than the inbred strains the general environment from which each strain was isolated weakly predicted its aggressiveness and plausibly suggests adaptation to growth in slowly rotting fruit or during wine fermentation favors more aggressive osmotic stress behaviors than growth in more severely hyperosmotic sugar cane syrups, molasses or olive wastes.

Classical evolutionary models assign fitness directly to genotypes, mutations, and mean trait values without consideration of the genotype-to-phenotype map; molecular models provide detailed mechanistic outlines of development but rarely consider the effects of natural genetic variation. The osmotic stress response is extensively characterized in a few strains and genotypes but until now osmotic stress signaling and behavior in a population had not been

examined. Labyrinthine developmental mechanisms, that are themselves controlled by genetic variation, translate genotypes into phenotypes with a variable fidelity that allows for the possibility of phenotypic heterogeneity and the evolution of bi-stable states[29]. Our view of osmotic stress signaling and response on a backdrop of natural variation enabled the identification of negative feedback controlling a robust recovery of steady-state viability in exponential growth. In post-diauxic cultures, continuous heritable variation in the distribution of cautious to reckless osmotic stress signaling is a risk spreading strategy. Microorganisms in nature spend a large amount of their time in post-diauxic or quiescent phases[21]. Our simple, 2-state model demonstrates how post-diauxic cells and strains can balance constraints between survival and evolutionary fitness through programmed bet hedging responses adapted to different patterns of environmental stress.

Materials and methods

Strain acquisition and deposition

Over 200 unique wild and industrial diploid strains of *Saccharomyces cerevisiae* were obtained from the fungal diversity collection of Centraalbureau voor Schimmelcultures (CBS), an institute of the Royal Netherlands Academy of Arts and Sciences in Utrecht, Netherlands (http://www.cbs.knaw.nl/index.php/collection/). Strains modified for this report are listed in Tables 1 and S1. They have been deposited to the Yeast Genetic Resources Lab of the National BioResource Project in Osaka, Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html/).

Haploid *MATa* library of wild and industrial genotypes

The first step in our library construction pipeline was to delete the *HO* locus of each strain by replacement with the KanMX4 marker gene and "barcodes" to permanently label each strain while preventing homothalism (Table S1)[30,31]. The KanMX4 gene was PCR-amplified for this purpose with primers containing the barcode sequences[32]. Next, kanamycin-resistant transformants were grown in pre-sporulation medium containing 10% glucose followed by sporulation under starvation conditions in 1% potassium acetate. Although the strains differ in their sporulation efficiency and optimal conditions (http://www.cbs.knaw.nl/Collections/), we found it was most efficient to put strains through repeated rounds of a general sporulation protocol rather than trying to optimize the conditions for each strain. The *MAT***a** haploids were identified by "schmoo" formation in 96-well plates containing alpha factor and confirmed by crossing to a G418-sensitive, clonNAT-resistant *MATalpha* tester strain and selection on double-antibiotic plates. Next we deleted the *URA3* gene using a standard gene deletion method and selected the *ura3Δ* clones by replica plating and selection on 5-FOA. Finally, *ho* and *ura3* deletions and the barcode sequences of each strain were verified by PCR and sequencing. Forty-nine wild strains and a laboratory strain meeting these criteria were used in this study (see Tables 1 and S1 for strain details).

Synthetic population of *GPD1*::GFP wild/lab diploids

The *MATalpha* laboratory strain BY4742 was transformed to create a stably integrated GPD1::*GFP* reporter (G01) using a deletion cassette containing a *URA3* marker for selection on SC-URA plates[32,33]. A synthetic "population" of diploids was created by mating each strain in the library of *MAT**a*** haploids (50 strains) with G01 by mixing on SC-URA plates for 2 hours followed by streaking onto selective SC-URA+G418 plates. The 50 resulting wild/lab diploid strains all have 50% of their genes from the *MATalpha GPD1*::GFP reporter in the BY4742 laboratory strain background (Table 1). After mating, it was necessary to screen for triploids or tetraploids, which express higher levels of *GPD1*::GFP and have higher tolerance to osmotic stress. Overnight cultures of wild/lab yeast were diluted 50-fold into fresh YPD+G418 and grown for an additional 4 hours, fixed by 1:3 dilution into cold ethanol and resuspended in 20 ug/ml RNAse A to digest ribonucleic acids. Digested cells were stained with 30 ug/ml propidium

iodide to label DNA and ploidy was determined by flow cytometry (FACS Calibur; Becton Dickinson).

Exponential and post-diauxic cultures

Fresh cultures were generated for each experiment by replicating frozen 96-well plates onto YPD+G418 agar followed by 4 days growth at 21° C. To obtain mid-exponential (ME) cultures, freshly patched cells were grown in 2 ml liquid YPD+G418 cultures at with rotation (72 rpm) at 21° C. for 2 days. Two microliters of these suspensions were diluted into 2 ml of liquid YPD+G418 and grown at 21° C. for 14 hours (e.g. 5 rounds of cell division on average, with strain ODs ranging from 0.80 – 1.44). For post-diauxic (PD) cultures, freshly patched cells were grown in 2 ml liquid YPD+G418 cultures at with rotation (72 rpm) at 21° C. for 4 days. Strains cultured up to 8-days post-diauxic growth were tested for osmotic stress resistance and we found that 4 day cultures were already maximally resistant (not shown).

Plating assays

To determine the adaptation limit of each strain, post-diauxic cultures were diluted to OD_{600} of 0.1 with exhausted YPD (to prevent re-growth), sonicated for 5 seconds at a low setting (2.5; Sonifier Cell Disrupter, Model W185) and plated (5 ul) on 96-well plates containing YPD without KCl (controls) or with KCl ranging from 2.0 to 3.0 M. Growth was tested after up to 2 months at 21° C. Viability and static survival under osmotic stress (Figures 2B and S2) was determined after incubation in liquid media with increasing concentrations of KCl followed by plating on iso-osmolar, YPD agar plates.

Microfluidics

We used custom made microfluidics devices with two fluid inputs as described[12]. When performing microfuidics with post-diauxic cells, post-diauxic cultures were inoculated into devices with exhausted YPD medium and allowed to stabilize for a few hours prior to osmotic stress. Experiments were run at ambient room temperature and observed using a Nikon TS100 inverted microscope. Recordings were made using a Photometrics CoolSnap HQ2 digital camera operated by Metavue (Molecular Dynamics). Analysis of acquired images was performed using Image J software (https://imagej.nih.gov/ij/).

Flow cytometry

For flow cytometry after osmotic stress 4 ml of PBS was added to each culture. Cells were isolated by centrifugation and resuspended in 1 ml PBS, transferred to FACS tubes, sonicated (5 seconds at level 3, Sonifier Cell Disrupter, Model W185) and stained with 3 ug/ml propidium iodide (PI) to monitor viability. After 20 min GFP fluorescence and viability were quantified using a FACS Calibur flow cytometer (Becton Dickinson) that had been calibrated prior to each use with SPHERO Rainbow Fluorescent Particles, 3.0 – 3.4 um (BD Biosciences). Flow

cytometry data were gated using magnetic windows in FlowJo software to eliminate cell fragments, clumped and dead (PI-positive) cells (http://www.flowjo.com/). Raw data for the viable cells in each sample (forward scatter, side scatter and GFP fluorescence data of up to 10,000 cells/sample) were extracted into an SQL database for analysis as described (https://figshare.com/s/52ef966b16cba7f41d7f/). Cell data were scaled for linearity (e.g. $FLH1^{1/3}$, $FSC^{1/3}$, $SSC^{1/2}$ for GFP fluorescence, forward scatter, and side scatter, respectively; see (https://figshare.com/s/8147275b62eb8d4db6bf/) for scalings and 3 dimensional (3-D) projections of cells). Distributions of *GPD1*::GFP accumulation in exponential cultures were unimodal and therefore well-defined using a single mean (e.g. Figure S1 and (https://figshare.com/s/8b709fd16cccbabc2a5a/)). By contrast, *GPD1*::accumulations of cells in post-diauxic cultures were clearly multimodal at many time points (Figure S3 and (https://figshare.com/s/8b709fd16cccbabc2a5a/). We identified Multi-normal distributions of cells based on 2-dimensional fits of *GPD1*::GFP and forward scatter data. The 2-dimensional fits distinguished different signaling slightly better than fitting *GPD1*::GFP only; adding side scatter to fit distributions in 3-dimensional space provided no additional resolution of cell types (see (https://figshare.com/s/8147275b62eb8d4db6bf/) for planar projections of each sample in 3 dimensions). Machine learning was performed on each sample using the sklearn.mixture option in the Gaussian Mixture Model (GMM) algorithm of the Python scikit package (http://scikit-learn.org/). The number of Gaussians to be fit is a parameter that must be provided to the model. We used Bayesian information criteria (BIC) to determine that the data were well described by four distributions. The GMM algorithm identified parameters of the four most-likely Gaussian (defined by means and covariances) given the data for each sample. In samples containing obviously fewer than four distributions, the under-populated distributions were assigned a correspondingly low frequency of cells (see Figures S1, S3 and associated links to complete data sets given above).

Clustering

To group, and ultimately rank, the strains according to their osmotic stress signaling responses to 2.5 M KCl during post-diauxic growth we used hierarchical clustering with Wards method in the fastcluster Python implementation (http://www.jstatsoft.org/v53/i09/)[34]. First we created state vectors of each strains behavior. Cell distributions were binned onto a 100 X 100 2-D grid according to their *GPD1*::GFP and forward scatter data, smoothed with Python scipy ndimage.filters.gaussian_filter and normalized to define a linear 1000 element state vector for each sample (strain, time point). While 100 bins on each axis where sufficient to capture detailed distributions while allowing efficient computation, we found stronger clustering when performing the same analysis using the combined *GPD1*::GFP and forward scattering data. The osmotic stress response up to 168 hours was defined by the vectors for each of the 7 time points, successively appended to form a 7,000 element time-line vector representing the combined evolution of *GPD1*::GFP accumulation and forward scatter data.

The time-line vectors were used to compute a distance matrix between strains using the symmetric Kullback-Leibler divergence. As each strains and time point was replicated between 4 and 15 times, we controlled for variation in sampling and clustering outcomes by randomly drawing samples for each strain and time point with equal probability. Clustering was repeated for a total of 17000 permutations requiring 43 hours of computation time on a 3.7 GHz Intel 7 iMac. This was sufficient to achieve stable Monte-Carlo statistics. Computational sorting of time-series distributions resolved 6 clades differentiated for rates of GFP accumulation, adaptation and survival.

Bet hedging model

Annotated code for our model of bet hedging with heritable probability binary cautious versus reckless bet hedging is publicly available (https://figshare.com/s/2c03544aef0c40cc86c2/). In brief, the bet hedging 'strategy' P was defined as the heritable probability of cautious cells for $0 \leq P \leq 1$. Relative fitness was measured for representative strategies (0, 0.1,0.2,…1.0) after 10 generations in each environment. Nine possible 2-state environmental shifts between three general osmotic stress environments were considered: permissive (E0; all cells grow equally well), restrictive (E1; reckless cells divide, cautious cells survive without dividing), and killing (E2; reckless cells die, cautious cells survive without cell division). For simplicity, the natural attrition of older cells (death and disappearance) and rates of cell division were assumed to be equal for all cell types. Results were independent of the number of generations in the first environment except as shown when E1 was the first environment.

References

1. Kirschner, M. & Gerhart, J. Evolvability. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8420–8427 (1998).
2. West-Eberhard, M. J. *Developmental plasticity and evolution*. *Nature* **424,** (2003).
3. Rutherford, S. L. From genotype to phenotype: buffering mechanisms and the storage of genetic information. *Bioessays* **22,** 1095–105 (2000).
4. Hohmann, S. Osmotic Stress Signaling and Osmoadaptation in Yeasts. *Microbiol. Mol. Biol. Rev.* **66,** 300–372 (2002).
5. Hohmann, S., Krantz, M. & Nordlander, B. *Yeast Osmoregulation*. *Methods Enzymol.* **428,** (Elsevier Masson SAS, 2007).
6. Saito, H. & Posas, F. Response to hyperosmotic stress. *Genetics* **192,** 289–318 (2012).
7. Clotet, J. & Posas, F. *Control of Cell Cycle in Response to Osmostress: Lessons from Yeast*. *Methods Enzymol.* **428,** (Elsevier Masson SAS, 2007).
8. Nadal, E. de, Alepuz, P. & Posas, F. Dealing with osmostress through MAP kinase activation. *EMBO Rep.* **3,** 735–40 (2002).
9. Babazadeh, R., Furukawa, T., Hohmann, S. & Furukawa, K. Rewiring yeast osmostress signalling through the MAPK network reveals essential and non-essential roles of Hog1 in osmoadaptation. *Sci. Rep.* **4,** 4697 (2014).
10. Ratcliff, W. C., Hawthorne, P. & Libby, E. Courting disaster: How diversification rate affects fitness under risk. *Evolution* 1–10 (2014). doi:10.1111/evo.12568
11. Remize, F., Barnavon, L. & Dequin, S. Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in Saccharomyces cerevisiae. *Metab. Eng.* **3,** 301–312 (2001).
12. Bennett, M. R. *et al.* Metabolic gene regulation in a dynamically changing environment. *Nature* **454,** 1119–1122 (2008).
13. Miermont, A. *et al.* Severe osmotic compression triggers a slowdown of intracellular signaling, which can be explained by molecular crowding. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 5725–30 (2013).
14. Lee, Y. J., Jeschke, G. R., Roelants, F. M., Thorner, J. & Turk, B. E. Reciprocal Phosphorylation of Yeast Glycerol-3-Phosphate Dehydrogenases in Adaptation to Distinct Types of Stress. *Mol. Cell. Biol.* **32,** 4705–4717 (2012).
15. Jacoby, T. *et al.* Two Protein-tyrosine Phosphatases Inactivate the Osmotic Stress Response Pathway in Yeast by Targeting the Mitogen-activated Protein Kinase, Hog1. *J. Biol. Chem.* **272,** 17749–17755 (1997).
16. Wurgler-Murphy, S. M., Maeda, T., Witten, E. a & Saito, H. Regulation of the Saccharomyces cerevisiae HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases. *Mol. Cell. Biol.* **17,** 1289–1297 (1997).
17. Muzzey, D., Gómez-Uribe, C. a., Mettetal, J. T. & van Oudenaarden, A. A Systems-Level Analysis of Perfect Adaptation in Yeast Osmoregulation. *Cell* **138,** 160–171 (2009).
18. Boy-Marcotte, E., Perrot, M., Bussereau, F., Boucherie, H. & Jacquet, M. Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in Saccharomyces cerevisiae. *J. Bacteriol.* **180,** 1044–52 (1998).
19. Yi, T. M., Huang, Y., Simon, M. I. & Doyle, J. Robust perfect adaptation in bacterial chemotaxis through integral feedback control. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4649–4653 (2000).
20. Galdieri, L., Mehrotra, S., Yu, S. & Vancura, A. Transcriptional Regulation in Yeast during Diauxic Shift and Stationary Phase. *Omi. A J. Integr. Biol.* **14,** 629–638 (2010).
21. Gray, J. V *et al.* 'Sleeping beauty': quiescence in Saccharomyces cerevisiae. *Microbiol. Mol. Biol. Rev.* **68,** 187–206 (2004).
22. Philippi, T. & Seger, J. Hedging one's evolutionary bets, revisited. *Trends Ecol. Evol. (Personal Ed.* **4,** 41–44 (1989).
23. Simons, A. M. Modes of response to environmental change and the elusive empirical evidence for bet hedging. *Proc. Biol. Sci.* **278,** 1601–1609 (2011).
24. King, O. D. & Masel, J. The evolution of bet-hedging adaptations to rare scenarios. *Theor. Popul. Biol.* **72,** 560–575 (2007).
25. Meyers, L. A. & Bull, J. J. Fighting change with change: Adaptive variation in an uncertain world.

Trends Ecol. Evol. **17,** 551–557 (2002).

26. Levy, S. F., Ziv, N. & Siegal, M. L. Bet hedging in yeast by heterogeneous, age-correlated expression of a stress protectant. *PLoS Biol.* **10,** (2012).
27. De Jong, I. G., Haccou, P. & Kuipers, O. P. Bet hedging or not? A guide to proper classification of microbial survival strategies. *BioEssays* **33,** 215–223 (2011).
28. Ratcliff, W. C. & Denison, R. F. Individual-Level Bet Hedging in the Bacterium Sinorhizobium meliloti. *Curr. Biol.* **20,** 1740–1744 (2010).
29. Rutherford, S. L. Between genotype and phenotype: protein chaperones and evolvability. *Nat. Rev. Genet.* **4,** 263–74 (2003).
30. Giaever, G. *et al.* Functional profiling of the Saccharomyces cerevisiae genome. *Nature* **418,** 387–391 (2002).
31. Shoemaker, D. D., Lashkari, D. a, Morris, D., Mittmann, M. & Davis, R. W. Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy. *Nat. Genet.* **14,** 450–456 (1996).
32. Wach, A., Brachat, A., Pöhlmann, R. & Philippsen, P. New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. *Yeast* **10,** 1793–1808 (1994).
33. Gietz, R. D. & Woods, R. a. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* **350,** 87–96 (2002).
34. Müllner, D. fastcluster : Fast Hierarchical , Agglomerative Clustering Routines for R and Python. *J. Stat. Softw.* **53,** 1–18 (2013).
35. Mortimer, R. K. & Johnston, J. R. Genealogy of principal strains of the yeast genetic stock center. *Genetics* **113,** 35–43 (1986).
36. Brachmann, C. B. *et al.* Designer deletion strains derived from Saccharomyces cerevisiae S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14,** 115–132 (1998).

Supplementary information and data deposition

Flow cytometry database
(https://figshare.com/s/52ef966b16cba7f41d7f/)
Python script for bet hedging model
(https://figshare.com/s/2c03544aef0c40cc86c2/)
Figure S1 complete data
(https://figshare.com/s/8b709fd16cccbabc2a5a/)
Figure S3 complete data
(https://figshare.com/s/8147275b62eb8d4db6bf/)

Acknowledgements

The wild strain collection was made and validated in the Rutherford lab over 3 summers with the help of talented teams of undergraduate lab assistants. We thank Wyming Lee Pang and Jeff Hasty for help with microfluidics and Stéphane Douady and Pierre-Yves Bourguignon for discussions and advice on scaling and clustering. This research was supported by a Scholar Award from the Damon Runyon Cancer Research Foundation (SR) and the Lady Tata Foundation (YH).

Contributions

SR designed experiments, wild haploid library construction and workflow, data analysis and writing. YH designed and performed experiments, data analysis, and writing. SB database construction, data analysis, statistical design and programming. WLP microfluidics design and training.

Author information

Current address for YH is Center for Experimental Animals, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan; WLP is UCSD Health Sciences, 9500 Gilman Drive, Dept 0012, La Jolla, CA 92093-0012.

None of the authors have competing interests.

Tables

Table 1. Strains and aliases used in this study. See Table S1 for details about each of the 49 wild haploid strain derivatives (WHXXX). For brevity, figures are labeled with the wild parent strain number (WXXX; see Table S1 for details).

Strain	Genotype	Source
WHXXX	*MAT**a** ura3Δ0 hoΔ::barcode::KanMX4*	Haploid *MAT**a*** isolates of wild strains. This study; see Table S1 for details.
BY41	*MAT**a** his3D1 leu2Δ0 met15Δ0 ura3Δ0 flo8-1 hoΔ::barcode::KanMX4*	Barcoded, *MAT**a*** derivative of BY4742 used as a control genotype for the laboratory strain background. This study.
BY4742 (BY01)	*MATalpha his3D1 leu2Δ0 lys2Δ0 ura3Δ0 flo8-1*	*MATalpha* laboratory strain. BY4741 and BY4742 backgrounds derive from a wild diploid isolated in Merced, California in 1938 on figs (EM93[35]; S228C[36]). They are distinguished primarily by the many generations it has been under laboratory selection.
G01	*MATalpha his3D1 leu2Δ0 lys2Δ0 ura3Δ0 flo8-1 gpd1D::GFP::URA3*	Used for monitoring GPD1. BY4742 background; this study.
WXXX.BY16	*MAT**a**/MATalpha LYS2/lys2Δ0 ura3Δ0/URA3 FLO8/flo8-1 hoΔ::barcode::KanMX4/ho*	Controls. A set of 49 wild/lab plus 1 BY41.BY16 control for effect of marker gene deletions. This study; see Table S1 for details.
WXXX.G01	*MATa/MATalpha HIS3/his3D1 LEU2/leu2Δ0 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 FLO8/flo8-1 hoΔ::barcode::KanMX4/ho GPD1/gpd1D::GFP::URA3*	Synthetic population of wild/lab diploids for GPD1 quantification. This study; see Table S1 for details.

Table 2. Negative feedback between rates of change in mean *GPD1*::GFP accumulation and viability among strains. To control for potential deviations from normality, parametric (Pearson's) and non-parametric (Spearman's) pairwise correlations between rates of change in *GPD1*::GFP accumulation and viability within (upper 3 rows) and between 2 hours time intervals (below). Changes occurring in earlier intervals, potentially causal for later changes, are listed first. As in Figure 1 all 50 strains were tested at 0, 2, and 4 hours and 18 strains were tested at 6 hours (mean values represent a minimum of 3 replicates per strain). Significant comparisons are indicated in bold.

N	Variable	Interval (hrs)	Variable	Interval (hrs)	Pearson's r	Spearman's r	Prob>|r| Pearson's	Prob>|r| Spearman's
50	ΔGPD1::GFP	0 – 2	Δviability	0 – 2	0.8235	0.7725	**<.0001**	**<.0001**
50	ΔGPD1::GFP	2 – 4	Δviability	2 – 4	0.7739	0.7217	**<.0001**	**<.0001**
18	ΔGPD1::GFP	4 – 6	Δviability	4 – 6	-0.2354	-0.1992	0.3470	0.4282
50	Δviability	0 – 2	ΔGPD1::GFP	2 – 4	-0.7867	-0.7411	**<.0001**	**<.0001**
50	Δviability	0 – 2	Δviability	2 – 4	-0.9670	-0.9503	**<.0001**	**<.0001**
50	ΔGPD1::GFP	0 – 2	ΔGPD1::GFP	2 – 4	-0.7685	-0.7697	**<.0001**	**<.0001**
50	ΔGPD1::GFP	0 – 2	Δviability	2 – 4	-0.8082	-0.7696	**<.0001**	**<.0001**
18	Δviability	0 – 2	Δviability	4 – 6	-0.1407	-0.2178	0.5777	0.3854
18	Δviability	0 – 2	ΔGPD1::GFP	4 – 6	-0.3704	-0.2549	0.1303	0.3073
18	ΔGPD1::GFP	0 – 2	Δviability	4 – 6	-0.0456	-0.0464	0.8573	0.8548
18	ΔGPD1::GFP	0 – 2	ΔGPD1::GFP	4 – 6	-0.4319	-0.4572	0.0735	0.0565
18	Δviability	2 – 4	Δviability	4 – 6	-0.0100	0.0733	0.9685	0.7726
18	Δviability	2 – 4	ΔGPD1::GFP	4 – 6	0.4316	0.3333	0.0737	0.1765
18	ΔGPD1::GFP	2 – 4	Δviability	4 – 6	0.1400	0.1207	0.5796	0.6332
18	ΔGPD1::GFP	2 – 4	ΔGPD1::GFP	4 – 6	0.3731	0.3602	0.1273	0.1421

Table 3. Growth of post-diauxic cells at unprecedented limits of adaptation. Shown are concentrations of agar media on which post-diauxic strains could grow and form colonies.

[KCl] M	Wild/lab (*GPD1*) diploids*
2.0	W455
2.6	W027, W035, W167, W202, W203, W242, W285, W454
2.7	W033, W041, W042, W134, W136, W150, W166, W178, W195, W215, W217, W219, W235, W248, W282, W291, W292, W294, BC41
2.8	W037, W044, W050, W153, W157, W163, W164, W179, W189, W206, W238, W244, W245, W249, W255, W276, W301, W340
2.9	W173, W211, W343

Table 4. Osmotic stress signaling behavior (rank) predicted early and late viability of post-diauxic cultures in osmotic stress. Least squares predictions of early and late viability by linear and 2nd order quadratic fits of fluorescence pre-accumulated into the G3 Gaussian at time 0 (G3_0) and ranked signaling behavior of 50 strains. The Bonferroni cutoff at the 0.05 level, based on 4 tests per data set, was 0.0125. Significant fits with lowest root mean squared errors and highest fraction of variation explained (R^2) shown in bold, predicted values for optimum (x) and value at optimum (y) for non-significant fits are marked NS.

	G_3 fluorescence (AU) at time 0		Signaling (rank)	
	quadratic	**linear**	**quadratic**	**linear**
0 hours, 0M KCl				
probability > F	**0.0006**	0.0016	0.9629	0.8454
R_square	**0.2715**	0.1891	0.0016	0.0008
root_mean_square_error	**1.3935**	1.4548	1.6313	1.6149
max_viability at optimum (%)	**98.8**		NS 98.2	
optimum AU or rank	**2626.6**		NS 31.9	
20 hours, 2.5M KCl				
probability > F	**< 0.0001**	< 0.0001	**0.0055**	0.6023
R_square	**0.4286**	0.3138	**0.1987**	0.0057
root_mean_square_error	**7.0949**	7.6940	**8.4022**	9.2615
max_viability at optimum (%)	**86.7**		**86.3**	
optimum AU or rank	**2662.5**		**24.4**	
48 hours, 2.5M KCl				
probability > F	**< 0.0001**	0.0003	**0.0013**	0.9159
R_square	**0.3148**	0.2371	**0.2464**	0.0002
root_mean_square_error	**7.6281**	8.1323	**8.1679**	9.3096
max_viability at optimum (%)	**82.2**		**82.9**	
optimum AU or rank	**2622.3**		**25.7**	
72 hours, 2.5M KCl				
probability > F	**0.0010**	0.0033	**0.0062**	0.8464
R_square	**0.2556**	0.1660	**0.1943**	0.0008
root_mean_square_error	**9.9877**	10.4608	**10.3903**	11.4501
max_viability at optimum (%)	**73.5**		**74.3**	
optimum AU or rank	**2586.8**		**25.1**	
96 hours, 2.5M KCl				
probability > F	**0.0060**	0.0018	**0.0065**	0.0954
R_square	**0.1956**	0.1862	**0.1927**	0.0569
root_mean_square_error	**8.4186**	8.3789	**8.4338**	9.0201
max_viability at optimum (%)	**71.7**		**71.0**	
optimum AU or rank	**3389.8**		**21.3**	
120 hours, 2.5M KCl				
probability > F	0.0791	0.0413	**0.0004**	0.0037
R_square	0.1023	0.0839	**0.2859**	0.1625
root_mean_square_error	8.5666	8.5636	**7.6405**	8.1881
max_viability at optimum (%)	NS 67.7		**74.7**	

optimum AU or rank	NS 1679		**18.1**	
144 hours, 2.5M KCl				
probability > F	0.3473	0.1675	**0.0047**	0.0501
R_square	0.0440	0.0393	**0.2038**	0.0776
root_mean_square_error	9.6837	9.6059	**8.8377**	9.4123
max_viability at optimum (%)	NS 61.9		**68.3**	
optimum AU or rank	NS 1481.3		**20.4**	
168 hours, 2.5M KCl				
probability > F	0.1785	0.3433	**<0.0001**	< 0.0001
R_square	0.0707	0.0187	**0.5733**	0.4911
root_mean_square_error	12.3795	12.5879	**8.3885**	9.0652
max_viability at optimum (%)	NS 56.2		**68.4**	
optimum AU or rank	NS 2077		**9.7**	
24 hours, 3M KCl				
probability > F	0.0107	0.0292	**< 0.0001**	< 0.0001
R_square	0.1757	0.0952	**0.5254**	0.2980
root_mean_square_error	11.8963	12.3332	**9.0267**	10.8635
max_viability at optimum (%)	71.7		**65.0**	
optimum AU or rank	2029.2		**18.1**	
48 hours, 3M KCl				
probability > F	0.0294	0.0140	**< 0.0001**	0.0003
R_square	0.1394	0.1193	**0.5382**	0.2402
root_mean_square_error	12.8903	12.9029	**9.4420**	11.9849
max_viability at optimum (%)	NS 57.1		**62.3**	
optimum AU or rank	NS 2867.3		**19.7**	
72 hours, 3M KCl				
probability > F	0.0519	0.0395	**< 0.0001**	0.0002
R_square	0.1183	0.0853	**0.5459**	0.2460
root_mean_square_error	12.4241	12.5218	**8.9158**	11.3687
max_viability at optimum (%)	NS 51.4		**56.9**	
optimum AU or rank	NS 2651		**19.7**	
Adaptation limit				
probability > F	0.5435	0.2699	**0.0002**	0.0086
R_square	0.0262	0.0258	**0.3099**	0.1379
root_mean_square_error	0.1344	0.1330	**0.1132**	0.1251
max_concentration optimum (M)	NS 2.8		**2.8**	
optimum AU or rank	NS -95.6		**31.2**	

607

608

609 **Table S1. Haploid derivatives of wild strains.** The source for all wild strains in this study
610 was the strain collection of the Royal Netherlands Academy of Arts and Sciences over the past
611 100 years (Table 1 and Table S1). This resource has been deposited at the Yeast Genetic
612 Resources Lab of the National BioResource Project in Osaka, Japan.
613

Alias	MAT	Genotype	Comments
WH027	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 459; isolated in 1938 in Castellina, Italy from grape must; barcode #29(Uptag sequence: GGCCCGCACACAATTAGGAA, Downtag sequence: GCGCCGCATTAACTAAACTA)
WH030	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1508; isolated in 1927 from starter for sorghum brandy; YH note: mating defective, hard to make wild/lab diploids; barcode #16(Uptag sequence: GTCCGAACTATCAACACGTA, Downtag sequence: GCGCACGAGAAACCTCTTAA)
WH033	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 405; isolated in 1925 in West Africa from catabo for billi wine, from Osbeckia grandiflora; barcode #187(Uptag sequence: CCGTGTACTGAATTACGATC, Downtag sequence: CCATCTTTGGTAATGTGAGG)
WH035	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 5822; isolated in 1967 from malt wine; barcode #30(Uptag sequence: GGTCTATGCAAACACCCGAA, Downtag sequence: GCCGTCTTGACAACCTTATA)
WH037	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1395; isolated in 1922 from an unknown source; barcode #235(Uptag sequence: GGCTAAGGGACAACACCTCA, Downtag sequence: GCCCGGCACATAGAAGTAAC)
WH041	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 5635; isolated in 1958 in South Africa from grape must; barcode #2(Uptag sequence: CCATGATGTAAACGATCCGA, Downtag sequence: TATATGGCAGCAGATCGCCG)
WH042	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 3081; isolated in 1958 in Spain from alpechin; barcode #12(Uptag sequence: GTGCGAACCAACGTACTACA, Downtag sequence: GCAGGAACACCACAGGGTTA)
WH044	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 422; isolated in 1926 in Odessa, Ukraine from beer; barcode #135(Uptag sequence: CCCGCGATTGTAATGAATAG, Downtag sequence: CATACTACGTGGGACAGTTG)
WH050	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 5112; isolated in 1962 in Spain from grape must; barcode #49(Uptag sequence: CTTACTGATAGCGTAGAGGT, Downtag sequence: GTGGTCTGCAAACCCAACAA)
WH134	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 2964; isolated in 1947 in Copenhagen, Denmark from distiller's rum yeast; barcode #18(Uptag sequence: GCCCTGATAACAAGGTGTAA, Downtag sequence: GCGCCTATTACACAAACGTA)

WH136	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 2811; isolated in 1957 from wine; wine yeast; barcode #20(Uptag sequence: GTGAGCGAAACACCGCGTAA, Downtag sequence: GGTAATACGCAACTCCTCTA)
WH150	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 2962; isolated in 1947 in Copenhagen, Denmark from distiller's rum; barcode #15(Uptag sequence: GCCGTAGCCACAAGAGTTAA, Downtag sequence: GCGGCCACTTACACAAATTA)
WH153	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 6458; isolated in 1972; barcode #37(Uptag sequence: GGGACCGCCAAAGCTATCAA, Downtag sequence: GTGAACAATAACGGCCTTGA)
WH157	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 6506; isolated in 1973 in UK brewery; killer yeast; barcode #53(Uptag sequence: CTGAGCGTAGGATATTCCGT, Downtag sequence: GCCGGTCGCAAACTCATAAA)
WH163	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 6914; isolated in 1977 in Spain from white wine; barcode #51(Uptag sequence: CTACGTCGGCTCATAGTCGT, Downtag sequence: GCTCTCGGCCAAGGAAACAA)
WH164	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 6978; isolated in 1984 from wine; wine yeast; barcode #59(Uptag sequence: CACTCGGATTCAGTTCTAGT, Downtag sequence: GGCCTTGCCAAACAGTCAAA)
WH166	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 7072; isolated in 1980 from distillate; barcode #62(Uptag sequence: CCTAGTTCGAGATTGCGAGT, Downtag sequence: GTGGTCGCCCAAGCAACAAA)
WH167	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 7173; isolated in 1985 from catabo for billi wine; wine yeast; barcode #43(Uptag sequence: CAGTATGCTAGATTCCGGGT, Downtag sequence: GTCCTCGCAAGAAAGGCCAA)
WH173	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 8615; isolated in 1998 in Italy from grape must; dry yeast for wine making; barcode #61(Uptag sequence: CCTGTAGTACGAGTATGAGT, Downtag sequence: GGTCTGCCCAAAGTCACAAA)
WH178	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1192; isolated in 1928 from wine; wine yeast; barcode #155(Uptag sequence: CGCACACGATTAAGGTCCAG, Downtag sequence: CACTGTTGGTAAGGTCTATG)
WH179	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1193; isolated in 1928 from wine; wine yeast; barcode #70(Uptag sequence: CAATAGGGTGTGACAGTTCT, Downtag sequence: CTACTTCGCGTGAGCTGGTT)
WH189	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1241; isolated in 1930 from an unknown source ; barcode #212(Uptag sequence: CCACTTAGTTCAATAGGCGC, Downtag sequence: CCGAGTATTACATTCTCACG)

WH195	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1256; isolated in 1937 from port wine; barcode #123(Uptag sequence: CGTGGAGCAGTTCGTATAAT, Downtag sequence: CTCGACGCTGGACGTTATGT)
WH202	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 7372; isolated in 1988; killer yeast, K2Rd (Young & Yagiu), K2R2 (Wickner); barcode #119(Uptag sequence: CAACGTAGAGTGAGGTACAT, Downtag sequence: CACTTAGCTTAGACTCGTGT)
WH203	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 7438; isolated in 1989 from wine; wine yeast; barcode #65(Uptag sequence: CTTTCGGACGTATGTGCAGT, Downtag sequence: CCTTGATGATAGAGGGCTTT)
WH206	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 7833; isolated in 1994 in Missouri, USA from lung of a man with immune deficiency syndrome; virulent strain; barcode #82(Uptag sequence: CATACAAAGAGAGGTGTCCT, Downtag sequence: CCCTTGCGATTGGTGCAGTT)
WH211	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 7838; isolated in 1994 in USA from patient; barcode #144(Uptag sequence: CGATACAAGTAAGTTGCGAG, Downtag sequence: CCTCTTACGAGATAGCGGTG)
WH215	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 7962; isolated in 1984 in Sao Paulo, Brazil from fermenting concentrated syrup from sugar cane; barcode #94(Uptag sequence: CCCGATTGAGGCATGGTTAT, Downtag sequence: CGCTTCGAGTATGGGATATT)
WH217	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 7964; isolated in 1995 in Sao Paulo, Brazil from fermenting concentrated syrup from sugar cane; barcode #92(Uptag sequence: CGCGGAGTATAGAGCTTTAT, Downtag sequence: CAATCGCTCGGAGGCGTATT)
WH219	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 439 ; isolated in 1933 in Lager Schartel, Germany from Silvaner grapes; barcode #93(Uptag sequence: CGACCCTGATGATCCTTTAT, Downtag sequence: CTACGGGCTCGATGCCTATT)
WH235	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 5952; isolated in 1968; barcode #102(Uptag sequence: GGCTACGATACATCTTCATC, Downtag sequence: CATTTGTAACCAGTTCGCTC)
WH238	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 6223; isolated in 1969 in Chile from grape juice; radiation resistant; barcode #104(Uptag sequence: CTATGTGCGGTAAGACGTAT, Downtag sequence: CGGCGTAGATTGTTAGCATT)
WH242	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 6333; isolated in 1942 in Costa Rica from rotting banana; Strain name NRRL Y-1350 (synonymous designation; NRRL YB-210, NRRL-210, NRRL-B210; Mortimer and Johnston (1986), Genetics 113: 35); barcode #56(Uptag sequence: CCTGTAGATTGACGTGTAGT, Downtag sequence: GCCCTCGTGACAAATCGAAA)

WH244	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 8266; isolated in 1996 from rotting fig; Strain name X2180, derived from S288C by self-diploidization (Mortimer and Johnston (1986), Genetics 113: 35). Did not survive freeze-drying.; barcode #122(Uptag sequence: CAGAGGGCACTGTTCTTAAT, Downtag sequence: CCCTGCTGTAGAGGTTATGT)
WH245	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 3000; isolated in 1956 in Pakistan from palm wine; wine yeast; barcode #138(Uptag sequence: CACATCGTTTAACACTGGAG, Downtag sequence: CTAGGAGGTTACAGTCATTG)
WH248	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 8049; isolated in 1981 from fish food; feed for fish and crustaceans; barcode #108(Uptag sequence: CGACCCGATGTAGTAGATAT, Downtag sequence: CCGCCGGATGTGATATAATT)
WH249	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 6069; isolated in 1981; hybrid strain (Y55-2 x JJ101); barcode #87(Uptag sequence: CACTGTGACCGAGGGATACT, Downtag sequence: CGCGCTATTATACTCGACTT)
WH255	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 3090; isolated in 1958 from white grape must; barcode #72(Uptag sequence: CACTGTGGACGATACGGTCT, Downtag sequence: CTGTACGTGCGATACTCGTT)
WH276	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1394; isolated in 1924 from pressed yeast; distillery yeast; barcode #176(Uptag sequence: CCACCGATGTAATTTGAGTC, Downtag sequence: CACTCTGCGTTAATGTTGGG)
WH282	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1460; isolated in 1927 in Indonesia from fermenting fruit; barcode #115(Uptag sequence: CATACTTAGGGATCAGGGAT, Downtag sequence: CCTTGTCTGAGAGCCGTTGT)
WH285	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1479; isolated in 1928 from wine; wine yeast; barcode #240(Uptag sequence: GCGGCCAATAGTAAACTTCA, Downtag sequence: GCCGCCGTGATAAGAAACAC)
WH291	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1576; isolated in 1931 in Sulawesi, Indonesia from sap of Arenga palm; barcode #117(Uptag sequence: CCTGAGGACTTATTCACGAT, Downtag sequence: CATTGGATTAGACCGTGTGT)
WH292	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1582; isolated in 1948 in Portugal from wine; barcode #118(Uptag sequence: CCGATTAGAGGTTGACAGAT, Downtag sequence: CACTGACTTCGAGGTCGTGT)
WH294	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1585; isolated in 1934 from sake-moto; sake yeast; barcode #180(Uptag sequence: CATTAAGGCGCACGTTTATC, Downtag sequence: CTATCCTAGAGATTTGAGGG)
WH301	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 1594; isolated in 1936 from juice of aren palm; barcode #182(Uptag sequence: CACGTTTGCGAATAGGTATC, Downtag sequence: CAGATACTATTAAGTGCCGG)

WH340	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 2805; isolated in 1954 from wine; wine yeast, particularly suitable for fruit wines; barcode #233(Uptag sequence: GCCGGGCTTAAATTGAATCA, Downtag sequence: GCTCCGACTGAAGAACTAAC)
WH343	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 2808; isolated in 1954 from grapes (Blauer Portugieser); wine yeast, suitable for fruit wines, yields more than 18% of alcohol; barcode #224(Uptag sequence: CCCGTGAATATAAGTGAAGC, Downtag sequence: CCTGGATTTGAAGCGTATAG)
WH454	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 6412; isolated in 1952 from sake; sake yeast; barcode #192(Uptag sequence: CCTTAGGGATAATGAGTTGC, Downtag sequence: CCAGTGTTCTAACGTGCAGG)
WH455	**a**	*MAT**a*** ura3Δ0 hoΔ::barcode::KanMX4	Original CBS #: CBS 440; isolated in 1934 in Taiwan from molasses; barcode #249(Uptag sequence: GCCCAGGCTAAATGTTAAGA, Downtag sequence: GAAGTACGCTCAAGACCGAC)
BC4741 (BY41)	**a**	MATa his3D1 leu2Δ0 met15Δ0 ura3Δ0 flo8-1 hoΔ::barcode::KanMX4	Original CBS #: Lab strain, BY4741; isolated in 1938 in Merced, CA, USA from rotting fig; derived from S288C, of which strain 88% of the gene pool is contributed by strain EM93 (Mortimer and Johnston (1986), Genetics 113: 35). Barcode #266(Uptag sequence: GGCCTAACTCAACAGACGGA, Downtag sequence: GCGCTCGACTAAGAGAAACC)

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Table S2. Clustering statistics used to rank signaling behavior. Statistics showing the fraction of 17,000 permutations in which strains were clustered with at least 50% of the other strains in each mean cluster. These data were used to rank total signaling behaviors from most cautious (1) to most reckless (50) based on the fraction of time each strain was associated with its mean cluster (characteristic of that cluster). See Figure 2.

Rank	Cluster	Strain	MC0	MC1	MC3	MC2	MC5	MC4
1	MC0	W455	0.8484	0.1516	0	0	0	0
2	MC0	W167	0.8365	0.1635	0	0	0	0
3	MC1	W219	0.6375	0.3625	0	0	0	0
4	MC1	W217	0.5536	0.4460	0.0004	0	0	0
5	MC1	W027	0.5214	0.4755	0.0028	0.0002	0	0
6	MC1	W042	0.4850	0.4892	0.0191	0.0044	0.0023	0.0001
7	MC1	W235	0.1976	0.5532	0.2266	0.0226	0	0.0002
8	MC1	W340	0.1700	0.5803	0.2354	0.0142	0	0
9	MC1	W454	0.3355	0.6610	0.0034	0	0	0
10	MC1	W134	0.1686	0.7376	0.0821	0.0117	0	0
11	MC1	W276	0.1617	0.7643	0.0658	0.0083	0	0
12	MC1	W294	0.1692	0.7834	0.0440	0.0034	0	0
13	MC1	W157	0.1195	0.7851	0.0866	0.0088	0	0
14	MC1	W202	0.1671	0.7855	0.0446	0.0027	0	0
15	MC1	W238	0.1337	0.8048	0.0580	0.0036	0	0
16	MC1	W035	0.1293	0.8086	0.0572	0.0050	0	0
17	MC1	W248	0.1494	0.8331	0.0172	0.0003	0	0
18	MC3	W130	0.0003	0.0671	0.7020	0.2307	0	0
19	MC3	W136	0	0.0092	0.6572	0.3334	0.0002	0
20	MC3	W203	0.0025	0.1348	0.6410	0.2214	0.0002	0
21	MC3	W285	0.0003	0.0806	0.6245	0.2924	0.0022	0
22	MC3	W163	0.0011	0.1474	0.6228	0.2285	0.0002	0
23	MC3	W206	0	0.0036	0.6203	0.3747	0.0014	0
24	MC3	BY41	0	0.0123	0.6102	0.3768	0.0008	0
25	MC3	W041	0.0002	0.0233	0.5625	0.4090	0.0050	0
26	MC3	W343	0.0012	0.0432	0.5522	0.3910	0.0123	0.0002
27	MC3	W292	0.0201	0.1962	0.4883	0.2893	0.0062	0
28	MC3	W189	0.0001	0.0205	0.4757	0.4715	0.0319	0.0003
29	MC3	W211	0	0	0.3698	0.5572	0.0696	0.0033
30	MC2	W245	0	0	0.2579	0.6898	0.0523	0
31	MC2	W291	0	0	0.2211	0.6786	0.0994	0.0009
32	MC2	W164	0	0	0.1517	0.6601	0.1852	0.0031

33	MC2	W249	0	0	0.3349	0.6417	0.0233	0.0001
34	MC2	W166	0	0	0.3372	0.6407	0.0220	0.0001
35	MC2	W179	0	0.0002	0.3569	0.6212	0.0217	0
36	MC2	W244	0	0	0.1478	0.6160	0.2225	0.0137
37	MC2	W173	0	0.0003	0.3801	0.6005	0.0188	0.0002
38	MC2	W215	0	0.0012	0.3162	0.6000	0.0815	0.0011
39	MC2	W037	0	0	0.0714	0.5308	0.3863	0.0115
40	MC2	W255	0	0.0011	0.4771	0.5141	0.0077	0
41	MC2	W050	0	0	0.0567	0.4672	0.4417	0.0343
42	MC5	W044	0	0	0.0021	0.0728	0.8501	0.0750
43	MC5	W195	0	0	0.0019	0.0744	0.8501	0.0736
44	MC5	W301	0	0	0.0029	0.0882	0.8203	0.0887
45	MC5	W150	0.0001	0.0004	0.0006	0.0414	0.7978	0.1598
46	MC5	W033	0	0	0	0.0279	0.7196	0.2525
47	MC5	W153	0	0	0	0.0181	0.6545	0.3274
48	MC4	W282	0	0	0	0	0.0596	0.9404
49	MC4	W242	0	0	0	0.0002	0.1477	0.8521
50	MC4	W178	0	0	0	0.0017	0.1697	0.8286

Table S3

Plate key

Left

	1	2	3	4	5	6
A	W027.BY01	W044.BY01	W153.BY01	W173.BY01	W203.BY01	W235.BY01
B	W033.BY01	W050.BY01	W157.BY01	W178.BY01	W206.BY01	W238.BY01
C	W035.BY01	W130.BY01	W163.BY01	W179.BY01	W211.BY01	W242.BY01
D	W037.BY01	W134.BY01	W164.BY01	W189.BY01	W215.BY01	W244.BY01
E	W041.BY01	W136.BY01	W166.BY01	W195.BY01	W217.BY01	W245.BY01
F	W042.BY01	W150.BY01	W167.BY01	W202.BY01	W219.BY01	W248.BY01

Right

	1	2	3
A	W249.BY01	W292.BY01	W455.BY01
B	W255.BY01	W294.BY01	
C	W276.BY01	W301.BY01	
D	W282.BY01	W340.BY01	
E	W285.BY01	W343.BY01	
F	W291.BY01	W454.BY01	

Figure legends

Figure 1. Rate of change in osmotic stress signaling with negative feedback predicts survival and robust recovery of exponential cultures in moderate osmotic stress.

A. Time course of mean accumulated *GPD1*::GFP fluorescence in exponential cultures exposed to 0.75 and 1.5 M KCl. Each point represents an independent replicate measurement; curves connect strain means at each time (with a minimum of 3 replicates for each point). In the absence of stress, all strains had high steady-state viability (propidium iodide dye exclusion; range 96.3 – 98.7%; mean 97.6%) and relatively low mean GFP fluorescence indicating low background activity of HOG pathway signaling through the GPD1 promoter and low *GPD1*::GFP accumulation (range 12.7 – 34.8 AU; mean 18.8 AU).

B. Pie charts show relative changes in mean viability (shaded area), mortality (white area) and *GPD1*::GFP accumulation (opacity level) after 2 hours in 0.75M KCl. Strains ordered by viability at 2 hours. The viability at 2 hours was proportional to the 2 hour viability of non-disrupted controls having two intact copies of the GPD1 gene (R^2 = 0.7085; P<0.0001; not shown).

C. Relationship between mean *GPD1*::GFP accumulation and viability in mid-exponential cultures exposed to 0.75 M KCl for 0, 2, 4, and 6 hours (h). Each data point represents the average of at least three replicates per strain (~10,000 cells/ sample). The ellipses indicate correlations between viability and fluorescence at alpha = 0.95. The inserts show relationships between changes in *GPD1*::GFP and viability over each time interval.

D. Plausible integral feedback drives a robust recovery of steady-state viability after 4 hours in 0.75 M KCl. Integrating feedback control would assure perfect adaptation of stress responses, water balance and steady state viability (included manuscript in preparation). By linear regression recovery = (0.7670) early mortality + 3.4936 (R^2 = 0.9351; P< 0.0001). Note that the persistence in cultures of dead cells over the course of the experiment precludes 100% recovery of steady-state viability, the data fit a model whereby surviving cells in adapted strains undergo 3 cell divisions.

Figure 2. Continuous variation in signaling behaviors and survival of postdiauxic cultures exposed to severe osmotic stress.

A. Strains classified by mean cluster (MC0 – MC5) and ranked top (1) to bottom (50) according to changes in GPD1::GFP accumulation over time (see Table S2 and methods). Each time point shows representative distributions of GPD1::GFP accumulation (green) and relative survival red (99.7% viability) to blue (11.7% viability). Cells above the 89th percentile (top 11%) are shown in black. Prior to osmotic challenge steady-state viabilities were uniformly high (range 93.0 – 99.6%; mean 98.2%). Rank-ordered mean clusters are topographically equivalent to a sequential ordering.

B. Relative viability of post-diauxic cultures (WXXX.BY01 controls) incubated in 3 M KCl before plating on iso-osmolar media. Cultures were re-ordered according to the ranked signaling behavior given in Figure 2A. The same experiment as originally plated is shown in Figure S1. Strains are color-coded as in Figure 1C for comparison of exponential and post-diauxic cultures.

Figure 3. A simple bet hedging model with heritable proportions of cautious and reckless cells produces observed variation in survival.

A. Cells with the most aggressive signaling began to die after long periods in severe stress leaving increasing fraction of cells with lower *GPD1*::GPF accumulations. Shown are distributions of accumulated *GPD1*::GFP and viability in replicate cultures of W242 (rank 49) in 5 replcate cultures after 168 hours in 2.5 M KCl. Mean (x), standard deviation (std), and weight (w; the fraction of cells in each distribution) are given. Sum (red) shows the cumulative fit of the 4 learned Gaussians.

B. Static viability of post-diauxic cells of strain W027 exposed to 3 M in microfluidic chambers. Individual cell behaviors mirror population behaviors measured by flow cytometry – e.g longer lag periods and increased accumulations of GPD1::GFP with increasing osmotic stress. Colored traces indicate accumulated fluorescence in representative cells in 1.5 (green), 2.0 (blue), 2.5 (red) and 3.0 M KCl (yellow). Arrows indicate average time to the first cell division +/- standard deviations.

C. A simple 2-state bet hedging model with heritable production of cautious (static) and reckless cell types. Bet hedging strategy P was defined as the probability of cautious cells for $0 \leq P \leq 1$. Relative fitness was measured for all strategies after 10 generations in each environment. All nine possible 2-state environmental shifts between three general osmotic stress environments were considered: permissive (E0; all cells grow equally well), restrictive (E1; reckless cells divide, cautious cells survive without dividing), and killing (E2; reckless cells die, cautious cells survive without cell division). Bet hedging and intermediate strategies ($0 < P < 1$) were most fit only when the environment shifted from moderate to more severe (E1 -> E2). When E1 was the first environment, the optimum strategy P depended on generation number.

Movie 1. Higher relative fitness of post-diauxic cells in extreme stress. Exponential W027.G01 seeded with a single post-diauxic cell of the same genotype (box). Cells were allowed to grow for 4 hours and then exposed to 1.5 M KCl at time 0h. Most cells rapidly die (as they fill with vacuoles) however the single post-diauxic cell expresses a high level of *GPD1*::GFP, adapts, and begins to divide.

Movie 2. Adaptation and growth of post diauxic cells. After about 4 hours in 1.5 M KCl W027.G01 express high levels of *GPD1*::GFP, adapt, and begin to divide. Cells that did not express *GPD1*::GFP accumulate vacuoles and die.

Movie 3. Reckless cells attempt to divide and fail in extreme osmotic stress. After 30 hours in 2.5 M KCl some post diauxic W027.G01 induce GFP to high levels, attempt cell division and pop, killing both mother and daughter. We think all strains have both cautious and reckless cell types; W027 (rank 5) is a relatively cautious strain.

Figure S1. Monophasic *GPD1*::GFP accumulations in exponential cultures is described well by distribution means. Representative samples of exponential cultures exposed to 0.75 M KCl for the times shown. Learned distributions of *GPD1*::GFP accumulation with mean (x), standard deviation (std), and weight (w; the fraction of cells in each distribution) are given (zero-weighted distributions not shown). Sum (red) shows the cumulative fit of the 4 learned Gaussians. The 18 representative strains are color-coded as in Figure 1B.

Figure S2. Static viability and survival of post-diauxic cultures in extreme osmotic stress. Strains were incubated for up to 5 weeks in 3 M KCl before plating on iso-osmolar media. A plate key is given in Table S3; the same data sorted by rank are shown in Figure 2B. This experiment, a qualitative assessment of viability, was repeated only once.

Figure S3a–c. Reproducibility of multiphasic distributions of *GPD1*::GFP in post diauxic cultures under severe osmotic stress. Representative replicates of post-diauxic cultures exposed to 2.5 M KCl for the times shown. Learned distributions of GPD1::GFP accumulation with mean (x), standard deviation (std), and weight (w; the fraction of cells in each distribution) are given (zero-weighted distributions not shown). Sum (red) shows the cumulative fit of the 4 learned Gaussians. The 18 representative strains are color-coded as in Figure 1B.

Figure S4. Rank predicts increasingly aggressive osmotic stress signaling behavior. The average percent of cells in each strain above a threshold set at the top 11% of accumulation of *GPD1*::GFP normalized across all post-diauxic cultures. Strains were exposed to 2.5 M KCl for increasing times shown and ordered according to rank. The number of strains in each mean clusters are indicated with increasingly lighter grey scale in their order of “recklessness” signaling (MC0, MC1, MC3, MC2, MC5, MC4). The numbers of strains in each mean cluster are 2 (MC0), 15 (MC1), 12 (MC3), 12 (MC2), 6 (MC5), 3 (MC4). The 18 representative strains are color-coded as in Figure 1B.

Figure S5. Mean cluster membership predicts viability over time in 2.5 M KCl. Shown are average viabilities among strains in each mean cluster and time point (shading). Asterisks

indicate significance at the ≤0.05 level by ANOVA or, where appropriate, Welch's ANOVA. The numbers of strains in each mean cluster are 2 (MC0), 15 (MC1), 12 (MC2), 12 (MC3), 3 (MC4), 6 (MC5; see Figure 2). Horizontal lines indicate the overall average viability (50 strains) at each time point.