Natural variation in hyperosmotic stress responses of budding yeast

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Abstract

Rapid cell division defines evolutionary fitness for microorganisms but can be lethal during stress. Cells stop dividing under osmotic imbalance while adjusting their internal turgor pressure to the environment. Here we describe trade-offs between osmotic stress signaling, survival, and proliferation in a synthetic population of 50, ecologically distinct budding yeast. During rapid growth strong signaling predicted survival. Weak signaling predicted low viability, intense rebound signaling, and robust recovery. By contrast older cultures survived and adapted to unprecedented osmotic stress with fitness depending on reproducible, strain-specific proportions of cells with divergent signaling strategies. The most 'cautious' cells survived extreme stress without dividing; the most 'reckless' cells attempted to divide too soon and failed, killing both mother and daughter. A simple model with heritable proportions of cautious and reckless cells generates tunable, rapidly diversifying bet hedging strategies that resemble natural variation and would evolve in response to different patterns of osmotic stress.

Classical evolutionary models assign fitness directly to genotypes, mutations, and mean trait values without consideration of the genotype-phenotype map. Yet labyrinthine developmental mechanisms, which are themselves controlled by genetic variation, translate genotypes into phenotypes with a variable fidelity that determines whether and how heritable differences translate into fitness differences[1]. The mapping between genetic variation and the spectrum of attributes and behaviors upon which selection acts defines population-level properties such as the capacity to evolve (evolvability), the capacity to withstand genetic and environmental perturbation (robustness or canalization), and the optimization, within a given genotype, of phenotypic responses across different environments (reaction norms)[2]. Mapping the effects of natural genetic variation on mechanisms of development and the expression of phenotypic variation is needed to understand the evolutionary trajectories of populations and species. 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 when the balance between external solutes and free water pressure in the cell can change suddenly[3]. 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[3–8]. In the wild, yeast and other microorganisms must balance their 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[9]. 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[6]. 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[3,5]. The sheer numbers of genes involved, their conservation and their elaborate circuitry suggest that a nuanced response to osmotic stress has been crucial and strongly selected throughout evolutionary history. The hyperosmotic stress response 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[3,5,6] making osmotic stress responses of budding yeast ideal for characterizing the mapping between signaling and behavior in a diverse population.

We measured osmotic stress signaling, survival and adaptation in exponentially growing and nearly quiescent cultures of diploid yeast[10]. Glycerol-3-phosphate dehydrogenase (*GPD1*) is a rate-limiting enzyme in glycerol production[11]. We used the synthesis and accumulation of green fluorescent protein (GFP) integrated into the *GPD1* gene (*GPD1*::GFP) 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 Mat

alpha) to a panel of wild and industrial genetic backgrounds –e.g. fifty diverse haploids of the opposite mating type extracted from globally diverse, sequence-validated *Saccharomyces cerivisiae* diploid strains deposited to the collection of the Royal Netherlands Academy of Arts and Sciences over the past 100 years (CBS; Table 1 and Table S1).

HOG signaling and behavior in young cultures

We followed the behavior of single cells before and after their exposure to osmotic stress using 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; Figure S1). 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 (Figure 1A). 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 (Figure1B 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 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 negative feedback controls are present in the osmotic stress response at many levels and timescales. For example, (1) unequal water pressures activate osmotic stress sensors, glycerol channels and other pressure-sensitive components that return to baseline when water balance is restored (e.g. see Figure 5 in Hohman 2002[3,5]), (2) GPD1p indirectly controls and is controlled by osmostress-sensitive kinases that respond to upward and downward changes in water balance[14],

113 and (3) nuclear Hog-1 MAP kinase increases the transcription of phosphatases that restore its
114 own cytoplasmic localization and basal activity[15–17]. In short negative feedback is present in
115 essentially all of the varied mechanisms that act, in concert, to increase intracellular glycerol and
116 restore water balance.
117 Consistent with acting negative regulation, there was a strong and highly significant
118 correlation between early mortality (0 – 2 hour decreases in viability) and later accumulations of
119 *GPD1*::GFP (2 – 4 hours; Table 2). We reasoned that cells and strains that adapt quickly
120 experience lower and less sustained effects of osmotic stress (e.g. water loss) with more rapidly
121 attenuated HOG pathway activity and lower *GPD1*::GFP accumulation. Conversely, surviving
122 cells of strains that were slower to adapt and less viable experience higher and more sustained
123 osmotic stress (and likely other stresses). Prolonged osmotic stress would sustain HOG
124 signaling and maintain *GPD1* transcription – which is also activated by general stress
125 responses[18] – further promoting *GPD1*::GFP accumulation (e.g. negative feedback regulation at
126 the level of general stress responses). Indeed, even as *GPD1*::GFP and viability became
127 negatively correlated, their *rates of change* remained positively correlated (2 – 4 hours; Table 2)
128 prompting a parsimonious and more widely accepted interpretation that osmotic stress signaling
129 promotes adaptation and viability during both the initial and recovery phases of the response
130 (Figure 1C, insets).
131 After 4 hours in 0.75M KCl all strains had adapted to a new steady state, and their
132 later viability remained largely unchanged (Figure 1C inset, lower right). Interestingly, the initial
133 decreases in steady-state viability (0 to 2 hour mortality) were almost perfectly restored by 4
134 hours (Figure 1D). And by 6 hours, early mortality and recovery were over 98% correlated (R^2 =
135 0.9852; not shown). The biological robustness of adaptation and recovery of steady state
136 viability in moderate hyperosmotic stress further supports the idea that negative feedback, acting
137 through continued activation of stress responses, drives the accumulation of *GPD1* and glycerol
138 in the surviving cells of less viable strains (and indeed, glycerol accumulation is a plausible
139 mechanism of integral feedback in the hyperosmotic stress response assuring perfect
140 adaptation[17,19]; see Figure 1E). However, despite their resilience in moderate osmotic stress, the
141 strains that were relatively slower to adapt would be ultimately less fit than rapidly adapting
142 strains due to their higher death rate, slower recovery, and lower steady-state viabilities before
143 and after hyperosmotic shifts.
144
145 **Extreme stress resistance of older cultures**
146 By contrast with exponential cultures, aging yeast cultures survived and adapted to long periods
147 in unprecedented osmotic conditions (Movies 1 and 2). As aging cultures reach confluence and
148 deplete the glucose in their media, stress response proteins accumulate and they undergo a
149 metabolic change called the diauxic shift[20]. During post-diauxic growth cell division slows and
150 then stops as cells enter quiescence[10]. Post-diauxic cultures survived up to 5 weeks in 3 M KCl
151 (41/50 strains) but they could not adapt and did not grow ('static viability'). When they were

plated on fresh isotonic media some cells in nearly every strain recovered rapidly and grew (Figure S2). When we tested their limits of adaptation on increasing concentrations of KCl three strains grew at 2.9 M KCl and all but one strain grew on 2.6 M KCl media (Table 3). We are unaware of previous studies reporting such extreme osmotic stress survival or adaption limits for budding yeast of any growth stage or genotype.

Heterogeneity of 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 size, shape and 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 also 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' cells failed to signal or divide but remained in a cellular state of static viability without dividing.

We used a Gaussian mixture model to assign the cells in each sample to four Gaussian distributions described by eight parameters – means and covariances – numbered according to increasing levels of fluorescence (G_0-G_3 distributions; Figure S3). Only G_{3_0}, that is *GPD1*::GFP pre-accumulated into cells of the G_3 distribution during post-diauxic growth *prior* to the osmotic challenge and therefore unrelated to osmotic stress signaling, predicted survival at any time. G_{3_0} predicted early but not later viability, and this relationship was better fit by 2nd order quadratic rather than linear functions of *GPD1*::GFP, suggesting early survival was higher in strains with intermediate G_3 level signaling (more variation explained and lower mean square errors; Table S3). Despite the fine-scaled characterization of osmotic stress signaling behaviors of different groups of cells in each strain, none of the distributions learned by the Gaussian mixture model – neither pre-accumulated G_{3_0}, total *GPD1*::GFP fluorescence, nor stress-induced *GPD1*::GFP in any distribution – embodied features of the osmotic stress signaling that were important for later survival.

Continuous variation in signaling behavior

In order to map osmotic stress-responsive signaling onto survival more directly we next quantified the osmotic stress signaling behaviors of the cells in each strain as they unfolded over time unbiased by Gaussian assumptions or approximations. First, normalized levels of induced *GPD1*::GFP were binned as in a histogram, creating a 'sample vector' of cell numbers in each of 100 successive intervals of fluorescence intensity. Next, sample vectors for increasing time points in each strain were linked to create time line vectors (700-mers). 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

191 times (mean clusters; see methods). Finally, the fraction of permutations in which each strain
192 grouped with more than half of the other strains in its mean cluster was used to rank that strain's
193 behavior relative to the other strains in its group (clustering statistics; Table 4).
194 The unique signaling trajectories of most strains were highly reproducible,
195 demonstrating the genetic basis of strain-specific proportions of cells with divergent behaviors
196 (Figure S3). Based on their shared and strain-specific signaling behaviors, the 50 strains in our
197 population rapidly converged onto two large groups made up of six mean clusters (Figure 2).
198 Each strain was further ordered within and between mean clusters and clades based on their
199 clustering statistics, with their rank order describing increasingly rapid accumulations of
200 *GPD1*::GFP and 'reckless' signaling (Figure S4). Both mean cluster and rank predicted viability
201 over time (Figure S5 and Table 4; respectively) thereby supporting the role of natural osmotic
202 stress signaling differences between strains in shaping variation in fitness during osmotic stress,
203 validating our method of clustering, and confirming the biological relevance of 'cautious' versus
204 'reckless' osmotic stress signaling.
205
206 **Evidence for bet hedging**
207 Bet hedging is defined as the expression of alternate, conditionally-adaptive phenotypes within a
208 clone of genetically identical organisms[9,21–24]. As cautious and reckless behaviors were found
209 both within and between strains, we wondered whether bet hedging could explain the observed
210 variation in signaling and survival. Indeed, different ranked signaling behaviors were also
211 adapted to different osmotic environments. Higher-ranked strains with more aggressive osmotic
212 stress signaling strategies were favored in milder conditions, but with increasing time in extreme
213 osmotic stress more cautious strains and behaviors became more fit (Table 4). For example,
214 W178 at rank 50 was most viable in moderate 0.75 M KCl, but in 2.5 M KCl the optimum rank
215 was 25 after 20 hours, 20 after 72 hours, and 9.6 after 168 hours (1 week). Viability decreased
216 most markedly among the most reckless strains (Figure S5). The changing relative fitness of
217 cautious versus reckless behavior in different environments provides strong empirical evidence
218 for bet hedging.[22]
219 To further link different bet hedging strategies with the degree and duration of osmotic
220 stress, we tested the idea that yeast experienced increasing stress over time in severe (2.5 M
221 KCl) osmotic conditions. We again incubated cultures for 168 hours in 2.5 M KCl, but they were
222 first exposed to a mild pre-stress (2 hours in 0.5 M KCl) to pre-induce osmotic stress proteins
223 and make them more resistant to subsequent stress. If optimum rank depended solely on time
224 independent of the degree of stress experienced by the different strains, then the optimum rank
225 should not change. However after the pre-stress viability increased by ~10% and optimum rank
226 shifted back toward more reckless behaviors (rank 9.6 to rank 18; $P<0.0001$), as expected if the
227 optimum rank was sensitive to the degree of stress experienced by the cells. After 168 hours in
228 2.5 M KCl, the most reckless cells began to selectively disappear from the highest-ranking
229 strains. Replicates with lower viability had fewer cells with high accumulations of *GPD1*::GFP,

230 smaller G3 distributions, and correspondingly larger distributions with lower mean *GPD1*::GFP
231 (Figure 3A). The loss of cells with the highest accumulations of *GPD1*::GFP could indicate that
232 *GPD1*::GFP levels decrease as cells begin to die, before they become permeable to vital dyes.
233 However, G3 distributions were stable over most time points and in most strains. More likely,
234 after 168 hours in 2.5 M KCl the most aggressive cells in the highest-ranking strains attempt to
235 divide prematurely and fail (e.g. Movie 3). Rapid signaling and adaptation, a fitness advantage in
236 milder conditions, becomes a liability in extremely severe or prolonged osmotic stress. On the
237 other hand static viability, evidenced in the survival of non-dividing cultures in 3 M KCl (Figure
238 3B) and directly observed by microscopy (Figure 2B), usually has dramatically reduced
239 evolutionary fitness, but in severe killing stresses it allows more cautious cells and strains to
240 survive.
241
242 **Evolution of bet hedging**
243 As different cells types within each strain were most viable under different conditions, we next
244 asked whether a simple, 2-state bet hedging model with heritable control of the relative
245 proportions of cautious and reckless cell types could account for the observed variation in
246 osmotic stress signaling and explain the complex relationship between rank and viability. If
247 reckless signaling and aggressive replication were default, ancestral behaviors, then cautious
248 signaling and behavior could have arisen as a later adaptation to unpredictable and potentially
249 lethal osmotic environments. We therefore assumed that the heritable probability of daughters
250 with cautious signaling and behavior (P) is the derived, bet hedging trait and ask whether it could
251 evolve.
252 We considered three very general osmotic stress environments that discriminated
253 between cautious and reckless cell types: (E0) a permissive environment in which both cautious
254 and reckless cells grow equally well, (E1) a restrictive environment approximating moderate
255 osmotic stress reckless cells divide and cautious cells survive without dividing, and (E2) a killing
256 osmotic stress where reckless cells die and cautious cells survive without cell division. The
257 relative fitness (cell numbers) of $0 \leq P \leq 1$ was calculated after several generations in each of 9
258 possible shifts between the three environments. Most environmental shifts favored optimum
259 strategies of either all cautious ($P = 1$) or all reckless cell types ($P = 0$; Figure 3D). Strictly
260 intermediate strategies ($0 < P < 1$) and bet hedging prevailed only when the osmotic environment
261 shifted from moderate to more severe, with the optimum P depending on the number of
262 generations in the first environment (E1 -> E2). A worsening osmotic environment was
263 experienced by cells in our experiments and would be common in nature. Shorter lag periods
264 and more cell divisions in E1 initially favor lower P and a higher proportion of reckless cells.
265 Longer lag periods (e.g. more severe conditions) and fewer cell divisions favor higher P and a
266 higher proportion of cautious cells. Our empirical data were consistent with this model –
267 lower-ranked strains with more cautious signaling behaviors, longer lag periods and fewer
268 attempted cell divisions became increasingly fit over time in extreme osmotic stress (Table 4).

While the simple 2-state model of bet hedging behaviors probably does not reflect the full complexity of each strain's signaling behavior, it provides a conceptual framework for understanding the observed variation in osmotic stress signaling strategies and generates testable hypotheses for further studies. Bet hedging in microorganisms has been thought to arise almost exclusively through stochastic switching with a low probability of alternate phenotypes[9,25]. By contrast the variation we observe in osmotic stress signaling and behavior is heritable, representing one of few examples known in a microorganism of the rapidly diversifying, within-clutch bet hedging strategy of plants and animals[9,25]. If the heritable probabilities of cautious cells and behavior we observed were sculpted by selection, milder and/or slowly worsening osmotic environments would generate more reckless strains (W242, rank 49 isolated from rotting banana; W282, rank 48 isolated from fermenting fruit) and more severe or rapidly deteriorating osmotic environments would generate cautious strains and behavior (e.g. W455, rank 1 from molasses; W217, rank 4 from sugar cane syrup). Indeed the general environments from which each strains wild or industrial genotype was isolated roughly predicted their aggressiveness but not their rank (for mean percent of cells above threshold; $P = 0.030$ by ANOVA). This bears deeper scrutiny, but suggests that growth on rotting fruit or in wine favored more aggressive osmotic stress signaling strategies than growth in more hyperosmotic sugar cane syrups, molasses or olive wastes.

In conclusion, we applied a standard laboratory assay of a well-characterized osmotic stress pathway across a population. By viewing strain specific differences in *GPD1*::GFP accumulation on the backdrop of natural variation in osmotic stress responses and survival we have been able to identify negative feedback in osmotic stress signaling and robust recovery during exponential growth. Yeast in nature spend most time in post-diauxic or quiescent phases[10]. In post diauxic cultures we identified natural variation in cautious to reckless bet hedging that was heritable and likely balances constraints between osmotic stress survival and evolutionary fitness. Combining our empirical measurements on each strain with a simple bet hedging model suggests that control over the probability of cautious signaling and behavior evolves in response to the frequency, intensity, and duration of episodic environmental stress.

Materials and methods

Strain acquisition and deposition

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).

Creation of haploid *MATa* library of wild and industrial genotypes

For unique identification of each strain and prevention of homothalism, the *HO* locus of each wild or industrial diploid strain was deleted and replaced with the KanMX4 marker gene and unique barcode sequences[26,27]. The KanMX4 gene was PCR-amplified for transformation of each strain with primers containing unique sets of barcode sequences[28] (Table S1). Kanamycin-resistant transformants were sporulated in 1% potassium acetate sporulation medium and *MAT**a*** haploids were selected by testing for schmoo formation in 96-well plates containing alpha factor. Mating type was confirmed by test mating with a dominantly marked *MATa* tester strain on replica plates. The *URA3* gene of the sporulated wild haploids (*MAT**a***) was deleted using standard gene deletion method. In brief, pJL164 plasmid DNA was transformed and cells were plated onto YPD plate grown for overnight at 30°C. Cells were replica plated on 5-FOA and grown for 2 days at 30°C. The *ho* and *ura3* deletions and barcode sequences were verified by PCR and resulting wild haploids were in many cases validated by their colony morphologies. Forty-nine wild strains and a laboratory strain met these criteria (see Tables 1 and S1 for strain details).

Construction of synthetic population of *GPD1*::GFP wild/lab diploids

The common laboratory strain BY stably integrated *GFP* reporter strain was created by replacing the *GPD1* gene with *GFP* gene using a deletion cassette containing a *URA3* marker for selection of transformants on SC-URA plates[28,29]. Wild *MATa* haploids were mated with *MATalpha* GFP reporter strains. Briefly, *MAT**a*** and *MATalpha* cells were mixed on SC-URA plates for 2 hours and streaked onto selective SC-URA+G418 plates. To test the BY4742 background for low function alleles that could critically compromise osmotic stress function, a homozygous laboratory strain was made by crossing BY4742a to cells of the opposite mating type to create a 50th strain in our population (BY4741alpha; Table 2).

Ploidy analysis

Polyploids had higher GFP and tolerance to osmotic stress. To screen for triploids or tetraploids obtained after mating, wild/lab yeast were inoculated into 2 ml YPD with appropriate antibiotics and cultured for 24 h at 21°C on rotator after which 40 ul of the cultured cells were then added to fresh 2 ml YPD and cultured for 4 h at 21°C on rotator. 800 ul of cells were fixed

339 with 1.7 ml cold ethanol and stored at -20°C at least 20 minutes, centrifuged at 2300 rpm for 5
340 min, washed with 100 mM Tris-HCl (pH 7.8) twice, and suspended in 500 ul of 100 mM Tris-HCl
341 (pH 7.8). To digest ribonucleic acids 10 ul of 10 mg/ml RNase A was added to cell suspension
342 and they were incubated for at least 60 min at 37°C. Digested cells were then centrifuged at
343 2300 rpm for 5 min and suspended in 500 ul of FACS buffer (0.5 M Tris-HCl, pH 7.5, MgCl,
344 NaCl.). To dissociate cell clumps cells, they were sonicated for 10 sec at setting 3 (Sonifier Cell
345 Disrupter, Model W185). Cells were stained with 30 ug/ml Propidium Iodide (PI). After leaving
346 them for 20 min in the dark, cells were examined for their ploidy with FACS Calibur (Becton
347 Dickinson).
348
349 **Plating assays**
350 Cells cultured in YPD with G418 for 4 d at 21 ^{o}C on rotator were diluted to OD_{600} 0.1 with
351 exhausted YPD and sonicated for 5 sec at setting 2.5. 5 ul of cells were plated on YPD with or
352 without KCl and incubated at 21 ^{o}C for 2 months. To determine adaptation limits at which strains
353 could resume growth in concentrations up to 3 M KCl, we inoculated PD cells in a 96-well plate,
354 agar media containing increasing concentrations of KCl ranging from 2 to 3 M. After 2-months
355 incubation at 21°C, we documented adaptation limit, the highest concentration of KCl in which
356 colonies could grow.
357
358 **Microfluidics**
359 We employed custom made microfluidics devices with two fluid inputs as described previously[12].
360 Experiments were run at ambient room temperature. Yeast cells were observed using Nikon
361 TS100 and recorded using a digital camera (Photometrics CoolSnap HQ2) operated by Metavue
362 (Molecular Dynamics). Analysis of acquired images was performed using Image J software.
363
364 **Flow cytometry**
365 After osmotic stress challenge 4 ml of PBS was added to each tube. Cells were isolated by
366 centrifugation and resuspended in 1 ml PBS, transferred to FACS tubes, sonicated, and stained
367 with 3 ug/ml propidium iodide (PI) to monitor viability. After 20 min GFP fluorescence and
368 viability were quantified in a FACS Calibur flow cytometer (Becton Dickinson). Data were
369 initially analyzed using magnetic windows in FlowJo software to eliminate cell fragments and
370 dead (PI-positive) cells.
371
372 **Flow cytometry data analysis**
373 Raw cell data were initially processed and trimmed to identify cells and the fraction of viable cells
374 using magnetic windows in FloJO data analysis software. Cell data for each sample including
375 forward scatter and GFP fluorescence data were then extracted into an executable SQL
376 database for analysis of single cell data. We performed multi-normal fits to extract quantitative
377 values of GPD1::GFP for the different cellular conditions of a strain at a given time point. The

exact number of distributions to be fit is a parameter that is not determined by the model and was determined from the data. We found that fitting both *GPD1*::GFP and forward scatter worked slightly better than *GPD1*::GFP in fitting the data, and that 4 distributions provided more stable fits than 3 distributions.< Figure S3- fit distributions G0-G4). Multi-normal fits were performed with the sklearn.mixture.GMM Gaussian Mixture Model algorithm of Python scikit package (http://scikit-learn.org/stable/index.html). The exact number of distributions to be fit is a parameter that is not determined by the model, not determined from the data. We found that fitting both *GPD1*::GFP and forward scatter worked slightly better than *GPD1*::GFP in fitting the data, and that 4 distributions was better (smaller ??) than 3 distributions.< Figure S3- fit distributions G0-G4).

Clustering

We used a clustering algorithm of *GDP1*::GFP accumulations to characterize the signaling behaviors of the strains over time. In brief, the *GPD1*::GFP distribution of each strain at every time point was converted into a vector of cell density (numbers of cells) in a small section (100 sections) of a normalized density plot of cells at each time point. Two-dimensional clustering on the Kullbak-Leibler distance function was used to follow the behavior of each strain over time. Strains and timepoints were replicated between 4 and 15 times. To control for variation in sampling and clustering outcomes samples were randomly drawn for each strain and time point with equal probability for 17000 permutations.

Strains were ranked from most static signaling to most aggressive, providing a continuous relationship between signaling, survival and adaptation with the number of permutations in which strains clustered with a majority of other strains in their clade was used to rank strains from the most static to the most aggressive. Computational sorting of time-series distributions resolved 6 clades differentiated for rates of GFP accumulation, adaptation and survival. We used a clustering algorithm of *GDP1*::GFP and forward scatter accumulations to characterize the signaling behaviors of the strains over time. We found that clustering based on both the combined *GPD1*::GFP and forward scatter data worked slightly better than only *GPD1*::GFP. In brief, for an experiment we consider for each cell the *GPD1*::GFP and forward scatter data. This 2D distribution of each strain at every time point was converted first into a 2D 100x100 grid with binned values, then into a normalized linear 1000 elements state vector characteristic for the *GPD1*::GFP and forward scatter data of cells at each time point. The vectors for all the *n* time points were then successively appended to form a nx1000 time line vector representing the time course of the combined GPD1::GFP and forward scatter data. The vectors were then used to compute a distance matrix between strains at each time point using a Kullback-Leibler divergence based distance function. We used hierarchical clustering with Ward"s method using the fastcluster implementation in Python[30](http://www.jstatsoft.org/v53/i09/). We found stronger cluster when performing the same analysis using the combined GPD1::GFP and forward scattering for each cell in the flow cytometry, and unless stated otherwise the

reported results are in this case. Data from experimental replicates were representatively sampled for each time point to generate a random average sampling of time line vectors for each strain.

Bet hedging model

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 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.

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509 **Supplementary information**

510 Python script for bet hedging model

511 Flow cytometry database

512 3 movies

513

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520

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525

526

527 **Tables**

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

531

Strain	Genotype	Source
WHXXX	*MATa ura3D0 hoD::barcode::KanMX4*	This study; haploid Mata isolates of wild strains. See Table S1 for details.
BY41	*MATa his3D1 leu2D0 met15D0 ura3D0 flo8-1 hoD::barcode::KanMX4*	Mata derivative of BY4742.
BY4742	*MATalpha his3D1 leu2D0 lys2D0 ura3D0 flo8-1*	This study; BY4741 and BY4742 backgrounds derive from a wild diploid isolated in Nerced, California in 1938 on figs (EM93[31]; S228C[32]). They are distinguished primarily by the many generations it has been under laboratory selection.
G01	*MATalpha his3D1 leu2D0 lys2D0 ura3D0 flo8-1 gpd1D::GFP::URA3*	This study; BY4742 background. For monitoring GPD1.
WXXX.BY16	*MATa/MATalpha LYS2/lys2D0 ura3D0/URA3 FLO8/flo8-1 hoD::barcode::KanMX4/ho*	This study; 49 wild/lab plus 1 BY41 control. See Table S1 for details.
WXXX.G01	*MATa/MATalpha HIS3/his3D1 LEU2/leu2D0 LYS2/lys2D0 ura3D0/ura3D0 FLO8/flo8-1 hoD::barcode::KanMX4/ho GPD1/gpd1D::GFP::URA3*	This study. 49 wild/lab plus 1 lab/lab diploids for GPD1 quantification. See Table S1 for details.

532

533 **Table 2. Negative feedback between rates of change in *GPD1*::GFP accumulation and**
534 **viability.** To control for potential deviations from normality, non-parametric pairwise
535 correlations between rates of change in *GPD1*::GFP accumulation and viability within (upper 3
536 rows) and between 2 hours time intervals (below). Changes occurring in earlier intervals, which
537 were potentially causal for later changes, are listed first. Results were qualitatively similar for
538 Pearson's correlations with all significant P-values remaining <0.0001. Significant comparisons
539 are indicated in bold.
540

Interval	Variable	Interval	Variable	Spearman's r	Prob>|r|
0 – 2 hours	**Δ*GPD1*::GFP**	**0 - 2 hours**	**Δviability**	**0.7725**	**<.0001**
2 – 4 hours	**Δ*GPD1*::GFP**	**2 - 4 hours**	**Δviability**	**0.7217**	**<.0001**
4 - 6 hours	Δ*GPD1*::GFP	4 - 6 hours	Δviability	-0.1992	0.4282
0 – 2 hours	**Δviability**	**2 - 4 hours**	**Δ*GPD1*::GFP**	**-0.7411**	**<.0001**
0 – 2 hours	**Δviability**	**2 - 4 hours**	**Δviability**	**-0.9503**	**<.0001**
0 – 2 hours	**Δ*GPD1*::GFP**	**2 - 4 hours**	**Δ*GPD*1::GFP**	**-0.7697**	**<.0001**
0 – 2 hours	**Δ*GPD1*::GFP**	**2 - 4 hours**	**Δviability**	**-0.7696**	**<.0001**
1 - 2 hours	Δviability	4 - 6 hours	Δviability	-0.2178	0.3854
1 - 2 hours	Δviability	4 - 6 hours	Δ*GPD1*::GFP	-0.2549	0.3073
1 - 2 hours	Δ*GPD1*::GFP	4 - 6 hours	Δviability	-0.0464	0.8548
1 - 2 hours	Δ*GPD1*::GFP	4 - 6 hours	Δ*GPD1*::GFP	-0.4572	0.0565
2 - 4 hours	Δviability	4 - 6 hours	Δviability	0.0733	0.7726
2 - 4 hours	Δviability	4 - 6 hours	Δ*GPD1*::GFP	0.3333	0.1765
2 - 4 hours	Δ*GPD1*::GFP	4 - 6 hours	Δviability	0.1207	0.6332
2 - 4 hours	Δ*GPD1*::GFP	4 - 6 hours	Δ*GPD1*::GFP	0.3602	0.1421

541
542

543 **Table 3. Growth of post diauxic cells at unprecedented limits of adaptation.** Shown are
544 concentrations of agar media on which post diauxic strains could grow and form colonies.
545

[KCl] M	Wild/lab (G*PD1*) 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

546
547

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. 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.

	G3_0 fluorescence (AU)		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	**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	**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	**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	**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	**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	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	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	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 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	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	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	NS -95.6		**31.2**	

558 **Table S1. Haploid derivatives of wild strains.** The source for all wild strains in this study
559 was the strain collection of the Royal Netherlands Academy of Arts and Sciences over the past
560 100 years (Table 1 and Table S1). This resource has been deposited at the Yeast Genetic
561 Resources Lab of the National BioResource Project in Osaka, Japan.
562

Alias	MAT	Genotype	Comments
WH027	a	MATa ura3D0 hoD::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	MATa ura3D0 hoD::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	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 5822; isolated in 1967 from malt wine; barcode #30(Uptag sequence: GGTCTATGCAAACACCCGAA, Downtag sequence: GCCGTCTTGACAACCTTATA)
WH037	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1395; isolated in 1922 from an unknown source ; barcode #235(Uptag sequence: GGCTAAGGGACAACACCTCA, Downtag sequence: GCCCGGCACATAGAAGTAAC)
WH041	a	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 3081; isolated in 1958 in Spain from alpechin; barcode #12(Uptag sequence: GTGCGAACCAACGTACTACA, Downtag sequence: GCAGGAACACCACAGGGTTA)
WH044	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 422; isolated in 1926 in Odessa, Ukraine from beer; barcode #135(Uptag sequence: CCCGCGATTGTAATGAATAG, Downtag sequence: CATACTACGTGGGACAGTTG)
WH050	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 5112; isolated in 1962 in Spain from grape must; barcode #49(Uptag sequence: CTTACTGATAGCGTAGAGGT, Downtag sequence: GTGGTCTGCAAACCCAACAA)
WH134	a	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 2811; isolated in 1957 from wine; wine yeast; barcode #20(Uptag sequence: GTGAGCGAAACACCGCGTAA, Downtag sequence: GGTAATACGCAACTCCTCTA)
WH150	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 2962; isolated in 1947 in Copenhagen, Denmark from ; distiller's rum yeast; barcode #15(Uptag sequence: GCCGTAGCCACAAGAGTTAA, Downtag sequence: GCGGCCACTTACACAAATTA)
WH153	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6458; isolated in 1972 location not reported; barcode #37(Uptag sequence: GGGACCGCCAAAGCTATCAA, Downtag sequence: GTGAACAATAACGGCCTTGA)
WH157	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6506; isolated in 1973 in UK brewery; killer yeast; barcode #53(Uptag sequence: CTGAGCGTAGGATATTCCGT, Downtag sequence: GCCGGTCGCAAACTCATAAA)
WH163	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6914; isolated in 1977 in Spain from white wine; barcode #51(Uptag sequence: CTACGTCGGCTCATAGTCGT, Downtag sequence: GCTCTCGGCCAAGGAAACAA)
WH164	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6978; isolated in 1984 from wine; wine yeast; barcode #59(Uptag sequence: CACTCGGATTCAGTTCTAGT, Downtag sequence: GGCCTTGCCAAACAGTCAAA)
WH166	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7072; isolated in 1980 from distillate; barcode #62(Uptag sequence: CCTAGTTCGAGATTGCGAGT, Downtag sequence: GTGGTCGCCCAAGCAACAAA)
WH167	a	MATa ura3D0 hoD::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	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1192; isolated in 1928 from wine; wine yeast; barcode #155(Uptag sequence: CGCACACGATTAAGGTCCAG, Downtag sequence: CACTGTTGGTAAGGTCTATG)
WH179	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1193; isolated in 1928 from wine; wine yeast; barcode #70(Uptag sequence: CAATAGGGTGTGACAGTTCT, Downtag sequence: CTACTTCGCGTGAGCTGGTT)
WH189	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1241; isolated in 1930 from an unknown source ; barcode #212(Uptag sequence: CCACTTAGTTCAATAGGCGC, Downtag sequence: CCGAGTATTACATTCTCACG)

WH195	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1256; isolated in 1937 from port wine; barcode #123(Uptag sequence: CGTGGAGCAGTTCGTATAAT, Downtag sequence: CTCGACGCTGGACGTTATGT)
WH202	a	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7438; isolated in 1989 from wine; wine yeast; barcode #65(Uptag sequence: CTTTCGGACGTATGTGCAGT, Downtag sequence: CCTTGATGATAGAGGGCTTT)
WH206	a	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 7838; isolated in 1994 in USA from patient; barcode #144(Uptag sequence: CGATACAAGTAAGTTGCGAG, Downtag sequence: CCTCTTACGAGATAGCGGTG)
WH215	a	MATa ura3D0 hoD::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	MATa ura3D0 hoD::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	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 5952; isolated in 1968; barcode #102(Uptag sequence: GGCTACGATACATCTTCATC, Downtag sequence: CATTTGTAACCAGTTCGCTC)
WH238	a	MATa ura3D0 hoD::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	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 8266; isolated in 1996 in 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	MATa ura3D0 hoD::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	MATa ura3D0 hoD::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	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 3090; isolated in 1958 from white grape must; barcode #72(Uptag sequence: CACTGTGGACGATACGGTCT, Downtag sequence: CTGTACGTGCGATACTCGTT)
WH276	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1394; isolated in 1924 from pressed yeast; distillery yeast; barcode #176(Uptag sequence: CCACCGATGTAATTTGAGTC, Downtag sequence: CACTCTGCGTTAATGTTGGG)
WH282	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1460; isolated in 1927 in Indonesia from fermenting fruit; barcode #115(Uptag sequence: CATACTTAGGGATCAGGGAT, Downtag sequence: CCTTGTCTGAGAGCCGTTGT)
WH285	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1479; isolated in 1928 from wine; wine yeast; barcode #240(Uptag sequence: GCGGCCAATAGTAAACTTCA, Downtag sequence: GCCGCCGTGATAAGAAACAC)
WH291	a	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1582; isolated in 1948 in Portugal from wine; barcode #118(Uptag sequence: CCGATTAGAGGTTGACAGAT, Downtag sequence: CACTGACTTCGAGGTCGTGT)
WH294	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1585; isolated in 1934 from sake-moto; sake yeast; barcode #180(Uptag sequence: CATTAAGGCGCACGTTTATC, Downtag sequence: CTATCCTAGAGATTTGAGGG)
WH301	a	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 1594; isolated in 1936 from juice of aren palm; barcode #182(Uptag sequence: CACGTTTGCGAATAGGTATC, Downtag sequence: CAGATACTATTAAGTGCCGG)

WH340	a	MATa ura3D0 hoD::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	MATa ura3D0 hoD::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	MATa ura3D0 hoD::barcode::KanMX4	Original CBS #: CBS 6412; isolated in 1952 from sake; sake yeast; barcode #192(Uptag sequence: CCTTAGGGATAATGAGTTGC, Downtag sequence: CCAGTGTTCTAACGTGCAGG)
WH455	a	MATa ura3D0 hoD::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 leu2D0 met15D0 ura3D0 flo8-1 hoD::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)

563

564

Table S2. Clustering statistics used to rank signaling behavior. Statistics showing the fraction of 17,000 permutations in which strains ranked with 50% of 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	

580 **Figure legends**

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

583 A. Time course of mean accumulated *GPD1*::GFP fluorescence in exponential cultures
584 exposed to 0.75 and 1.5 M KCl. Each point represents an independent replicate
585 measurement; curves connect strain means at each time. In the absence of stress, all
586 strains had high steady-state viability (propidium iodide dye exclusion; range 96.3 –
587 98.7%; mean 97.6%) and relatively low mean GFP fluorescence indicating low
588 background activity of HOG pathway signaling through the GPD1 promoter and low
589 *GPD1*::GFP accumulation (range 12.7 – 34.8 AU; mean 18.8 AU).

590 B. Pie charts show relative changes after 2 hours in 0.75M KCl of mean viability (shaded
591 area), mortality (white area) and *GPD1*::GFP accumulation (percent opacity). Strains
592 ordered by viability at 2 hours, which was proportional to viability of non-disrupted
593 controls having two intact copies of the GPD1 gene (R^2 = 0.7085; P<0.0001;).

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

599 D. Robust recovery of steady-state viability after 4 hours in 0.75 M KCl driven by negative
600 feedback (see text; recovery = (0.7670) early mortality + 3.4936; R^2 = 0.9351; P<
601 0.0001).

602 E. Plausible integrating negative control via multiple feedback mechanisms resulting in
603 accumulation of intracellular glycerol. Integrating feedback control would assure perfect
604 adaptation of stress responses, water balance and steady state viability. Note that the
605 persistence of dead cells over the course of the experiment precludes 100% recovery.

606

607 **Figure 2. Signaling behaviors predict the survival and fitness of older cultures in severe**
608 **osmotic stress.**

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

616 B. B. Relative viability of post-diauxic cultures (WXXX.BY01 controls) incubated in 3 M KCl
617 before plating on iso-osmolar media. Cultures were re-ordered according to the ranked
618 signaling behavior given in Figure 2A. The same experiment as originally plated is shown

in Figure S1. Strains color coded as in Figure 1C for comparison of exponential and post diauxic cultures.

Figure 3. Extreme cautious and reckless cell types observed in post diauxic cultures produce observed variation in cautious to reckless signaling strategies in a simple bet hedging model.

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) 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. Generation of intermediate optima in 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 environmental shifts are shown. 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). Only in shifts from moderate to more severe (E1 -> E2) do bet hedging, intermediate strategies ($0 < P < 1$) prevail. The optimum strategy P depends on the number of generations in the first moderate stress environment. Results were independent of the number of generations in the first environment except as shown when E1 was the first environment.

Figure S1. Monophasic signaling behavior of exponential cultures described well by mean *GPD1*::GFP accumulations at each time point.

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. The representative strains shown are color-coded as in Figure 1B.

Figure S2. Static viability and survival of post diauxic cultures in extreme osmotic stress.

658 Strains were incubated for up to 5 weeks in 3 M KCl plated on iso-osmolar media. A plate key
659 is given in Table S3. The same data are shown sorted by rank in Figure 2B.
660
661 **Figure S3a–c. Reproducibility of cell distributions in strains with cautious to reckless**
662 **signaling.** Learned distributions of GPD1::GFP accumulation for post diauxic strains exposed
663 to 2.5 M KCl for the times shown. Mean (x), standard deviation (std), and weight (w; the
664 fraction of cells in each distribution) are given. Sum (red) shows the cumulative fit of the 4
665 learned Gaussians. The representative strains shown are color-coded as in Figure 1B.
666
667 **Figure S4. Rank predicts increasingly aggressive osmotic stress signaling.** Rank
668 predicts the relative proportion of cells above a threshold set at the top 11% of accumulation of
669 *GPD1*::GFP in post diauxic cultures exposed to 2.5 M KCl for the times shown. Mean clusters
670 are indicated with increasingly lighter grey scale in order of signaling (MC0, MC1, MC3, MC2,
671 MC5, MC4).
672
673 **Figure S5. Mean clusters were differentiated for predicts viability of post diauxic cultures.**
674 Mean cluster predicted viability. Shown are average viabilities each mean cluster were
675 analyzed by ANOVA or where appropriate by Welch's ANOVA at each time. Asterisks indicate
676 significance at the ≤0.05 level. Decreasing mean viabilities at each time are indicated
677 (horizontal lines).
678