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An Evolutionarily Conserved Prion-like Element Converts Wild Fungi from Metabolic Specialists to Generalists

Daniel F. Jarosz,[1,2,7] Alex K. Lancaster,[1,3,4,7] Jessica C.S. Brown,[1,5,8] and Susan Lindquist[1,5,6,*]
[1]Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA
[2]Departments of Chemical and Systems Biology and of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, USA
[3]Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA
[4]Center for Biomedical Informatics, Harvard Medical School, Boston, MA 02115, USA
[5]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[6]Howard Hughes Medical Institute, Chevy Chase, MD 20815, USA
[7]Co-first author
[8]Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94158, USA
*Correspondence: lindquist_admin@wi.mit.edu
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SUMMARY

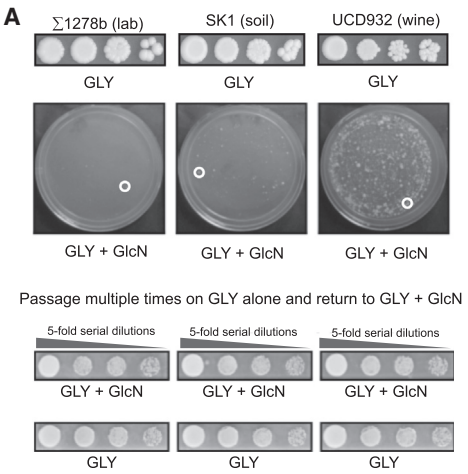
[*GAR*$^+$] is a protein-based element of inheritance that allows yeast (*Saccharomyces cerevisiae*) to circumvent a hallmark of their biology: extreme metabolic specialization for glucose fermentation. When glucose is present, yeast will not use other carbon sources. [*GAR*$^+$] allows cells to circumvent this "glucose repression." [*GAR*$^+$] is induced in yeast by a factor secreted by bacteria inhabiting their environment. We report that de novo rates of [*GAR*$^+$] appearance correlate with the yeast's ecological niche. Evolutionarily distant fungi possess similar epigenetic elements that are also induced by bacteria. As expected for a mechanism whose adaptive value originates from the selective pressures of life in biological communities, the ability of bacteria to induce [*GAR*$^+$] and the ability of yeast to respond to bacterial signals have been extinguished repeatedly during the extended monoculture of domestication. Thus, [*GAR*$^+$] is a broadly conserved adaptive strategy that links environmental and social cues to heritable changes in metabolism.

INTRODUCTION

To prosper in changing environments, organisms must have the capacity to acquire new, heritable phenotypes. It is a textbook assumption that such phenotypic diversity is achieved through genetic mutations. Prions and other epigenetic mechanisms provide an entirely different route to achieving heritable phenotypic diversity. Specifically, self-perpetuating changes in biological functions are passed from mother cells to their daughters without corresponding changes in DNA.

As generators of heritable diversity, prions and other epigenetic elements contrast with DNA-based mutations in at least two ways. First, cells lose these elements at much higher frequencies than mutations revert to wild-type. This prevents a phenotypic "lock-in" should the environment change to disfavor the epigenetic state. In rapidly changing environments, adaptive mutations can "strand" the population if the environment again changes to disfavor that phenotype. Second, environmental stresses can increase the rate at which cells acquire (and lose) epigenetic elements (Tyedmers et al., 2008; Newnam et al., 2011; Chernova et al., 2011; Holmes et al., 2013; Cox et al., 1988). In the case of yeast prions, this is because suboptimal growth conditions stress the cellular protein-folding network, and prion induction and inheritance are affected by alterations in protein folding (Shorter and Lindquist, 2005, 2008; Balch et al., 2008). In the case of human cancers, this is because diverse stresses of malignancy induce chaperones and chromatin-modifying enzymes that empower the epigenetic inheritance of cancer phenotypes (Kaelin and McKnight, 2013; Dawson and Kouzarides, 2012; Lu and Thompson, 2012). Epigenetic mechanisms therefore provide a general means through which cells "hedge their bets" precisely when their phenotypes are ill-suited to their environment. Although organisms can increase mutation rates in response to stress, these mechanisms are largely confined to responses directly tied to DNA metabolism (e.g., stalled replication forks during nucleotide starvation). Thus, epigenetic mechanisms for creating heritable forms of phenotypic diversity might confer an advantage over genetic mutations in fluctuating environments (Shorter and Lindquist, 2005; Halfmann et al., 2010; Newby and Lindquist, 2013).

Heritable epigenetically generated phenotypic diversity provides a route to the rapid creation of complex traits. However, a key prediction for an adaptive mechanism of this type is that its switching rates should be tuned to the organism's particular ecological niche (Lachmann and Jablonka, 1996; Kussell and Leibler, 2005; Lancaster and Masel, 2009; Lancaster et al.,



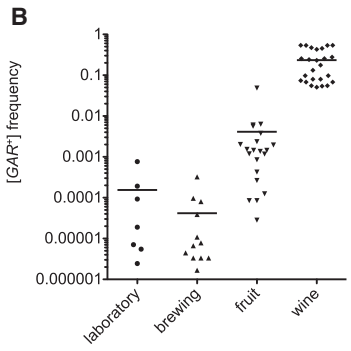


Figure 1. [*GAR*$^+$] Is Common in Wild Strains of *S. cerevisiae*

(A) Diverse wild strains of *S. cerevisiae* have the capacity to acquire the heritable ability to grow on GLY + GlcN.

(B). Scatterplot of the frequency of [*GAR*$^+$] appearance among *S. cerevisiae* strains from different ecological niches

See also Figure S1 and Table S1.

2010). The vast majority of epigenetic mechanisms for phenotypic diversification have not been shown to fulfill this criterion (de Jong et al., 2011). Moreover, in microorganisms, there is no evidence that any such strategy has been conserved through evolution for the competitive advantages it provides for life in dynamic natural communities.

A recently discovered yeast epigenetic element, [*GAR*$^+$], provides a particularly interesting subject for investigation. Although its biochemical underpinnings are complex, [*GAR*$^+$] has many properties of a yeast prion. It arises at a frequency higher than expected for mutations, it is dominant, it shows non-Mendelian inheritance in genetic crosses, and its transfer from one generation to the next relies upon the activities of a molecular chaperone. [*GAR*$^+$] therefore has the defining genetic features of prion-based inheritance.

Biologically, [*GAR*$^+$]'s effects are simple and robust: it circumvents one of the central metabolic properties of yeast, glucose repression (Brown and Lindquist, 2009). This ancient regulatory mechanism prevents *Saccharomyces cerevisiae* from metabolizing most carbon sources in the presence of even trace amounts of glucose. Because yeast cells have an extreme preference for glucose, they are metabolic "specialists." In the presence of glucose, they ignore virtually all other carbon sources and maximize the production of carbon dioxide and ethanol. It is this trait that has motivated man's pervasive exploitation of *S. cerevisiae* (Rozpędowska et al., 2011) for the production of alcoholic beverages. The [*GAR*$^+$]-driven switch in metabolism circumvents this trait, allowing yeast to become metabolic "generalists" and utilize multiple carbon sources in the presence of glucose (Jarosz et al., 2014 [this issue of *Cell*]).

Although yeasts are typically cultured on pure sugars in the laboratory, this epigenetic switch in metabolic lifestyle might provide adaptive value in natural environments, where yeast frequently encounter mixed carbon sources (Bisson et al., 2007). In the accompanying paper, we report that [*GAR*$^+$] also provides adaptive value when yeast cells are grown in the presence of bacteria. The prion is induced by a chemical signal secreted by evolutionarily diverse bacteria and is the only prion currently known to be induced in response to any other organism. The bacteria thrive when yeast acquire [*GAR*$^+$] because the yeast produce less ethanol, providing a less hostile environment. Yeast likewise benefit, gaining the ability to metabolize mixed carbon sources, improved nutrient uptake capacity, and extended lifespan (Jarosz et al., 2014).

Here we investigate the adaptive significance of [*GAR*$^+$]-based metabolic switching. We ask whether switching rates vary with the diverse ecological niches yeast occupy and whether [*GAR*$^+$] is naturally present in wild *S. cerevisiae* isolates. We quantitatively investigate the adaptive value of this epigenetic reversal of glucose repression in evolutionarily diverse wild fungi. We explore the evolutionary breadth of the [*GAR*$^+$] phenotype and its regulation by secreted bacterial factors. Finally, we test the hypothesis that [*GAR*$^+$] has been selected for life in social communities by examining its extinction during domestication.

RESULTS

The Circumvention of Glucose Repression Correlates with Ecological Niche

To assess the potential adaptive value of [*GAR*$^+$], we first asked whether the rate at which yeast cells switch between heritable glucose repressed and glucose derepressed states varies with the ecological niche from which they were isolated. We analyzed multiple individual colonies of ~100 genetically and ecologically diverse wild *S. cerevisiae* strains obtained from stock centers (Table S1 available online). The strains had been archived after a minimal number of generations in culture to preserve biological characteristics selected for in their natural niches.

We suspended and grew these strains for a few generations in rich liquid glucose medium and compared the frequencies at which they spontaneously acquired a heritable [*GAR*$^+$]-like state. To do so, we plated cells onto glycerol medium (GLY), with and without trace quantities of glucosamine (GlcN). GlcN is structurally very similar to glucose, but it cannot be metabolized by yeast. GlcN therefore provides a stable signal that glucose is present in the culture and triggers glucose repression. GlcN thereby prevents yeast cells from growing on glycerol. However, cells that acquire [*GAR*$^+$] can circumvent this repression and grow robustly on GLY + GlcN medium (Brown and Lindquist, 2009).

Glucose repression is generally considered a defining characteristic of *S. cerevisiae*. As expected, wild *S. cerevisiae* strains from diverse ecological niches could grow well on GLY medium but could not grow on GLY + GlcN (Figure 1A). However, in each strain, variants appeared that could grow on this medium

(Figure 1A). Remarkably, the frequency with which such variants appeared ranged over five orders of magnitude.

These differences in frequency were a stable characteristic of each strain. Moreover, they varied in a manner that correlated with ecological niche (Figure 1B). Colonies that could grow on GLY + GlcN appeared in all 14 brewery strains we tested at frequencies similar to those of most laboratory strains (between one in 50,000 to one in 10,000 cells). The trait appeared with much higher frequencies in all 21 strains isolated from fruit (roughly 1 in 50 to 1 in 500 cells). Wine strains had the highest frequencies. As many as 1 in five of such glucose-grown cells had the ability to grow into a colony on GLY + GlcN medium.

All of these variants retained the capacity to grow immediately and robustly on GLY + GlcN after multiple passages on nonselective glucose medium (Figure 1A). This constituted many hundreds of mitotic cell divisions. That is, once this new metabolic trait appeared, it was transmitted from one generation to the next even in the absence of any selective pressure.

Importantly, genetically distant strains from the same niche acquired the ability to grow on GLY + GlcN at strikingly similar frequencies. For example, the fruit strains DBVPG1106, UWOPS83_787, UWOPS03_461, and UWOPS05_217 had similar frequencies despite their pronounced evolutionarily divergence (Figure S1). Moreover, in genetically similar strains adapted to different niches, the ability to grow on GLY + GlcN appeared at very different frequencies. For example, the genetically closely related strains Y9 (isolated from sake) and K11 (isolated from ragi) differed by several orders of magnitude (Figure S1). Overall our analysis of these and other sequenced wild strains suggests that it is not common ancestry but the ecological niche that is most important in determining the rate at which this trait appears (Figure S1).

The Heritable Circumvention of Glucose Repression in Wild Strains Is due to [*GAR*$^+$]

The ability to grow on GLY + GlcN can be acquired in laboratory strains through genetic mutations, but these are all recessive (Ball et al., 1976; Kunz and Ball, 1977). The wild strains we examined were all diploid (or polyploid). Therefore, the frequency at which cells acquired the ability to grow on GLY + GlcN made it extremely unlikely that the trait arose from de novo mutations. Because prion inheritance is based upon self-templating protein conformations, prion phenotypes are dominant (Shorter and Lindquist, 2005). Spontaneous appearance of the [*GAR*$^+$] prion would therefore provide an attractive explanation for the frequent and highly variable spontaneous appearance of this trait. To investigate this possibility, we tested 20 variants that could grow on GLY + GlcN plates—garnered from strains representing each of the diverse ecological niches—for several hallmarks of [*GAR*$^+$] cells.

When cells switch from the [*gar*$^-$] to [*GAR*$^+$] state, transcription of the *HXT3* gene is strongly repressed (Brown and Lindquist, 2009). We used this change in gene expression as a test for [*GAR*$^+$] because genetic manipulation of other factors involved in the prion phenotype produces many pleiotropic effects and can be technically challenging in wild diploid strains. Each of the original *S. cerevisiae* ecotypes had high levels of *HXT3* mRNA. Even when growing on glucose, all of the variants that had spontaneously acquired the ability to grow on GLY + GlcN had low levels of this transcript (Table S2).

Next, we examined a property common to most known prions. When prions appear de novo, they produce a spectrum of phenotypes from "strong" to "weak," and these phenotypes are faithfully propagated from one generation to the next. When [*GAR*$^+$] arises in laboratory strains, it also produces strong phenotypes (robust growth on GLY + GlcN) and weak phenotypes (moderate growth on GLY + GlcN; Brown and Lindquist, 2009). Similar variants appear in cells derived from each of the ecological niches, and these distinct states are stable through many rounds of passage on nonselective media (data not shown).

Finally, we applied a genetic test for [*GAR*$^+$] inheritance that was possible even in wild strains, which are much less genetically tractable than laboratory strains. Because prions are based on the inheritance of protein conformations, transient changes in protein-folding functions produce heritable changes in prion phenotypes. Other well-characterized prions are particularly sensitive to changes in Hsp104 activity, but [*GAR*$^+$] inheritance is uniquely sensitive to changes in the protein chaperone known as Hsp70 (particularly Ssa1; see Brown and Lindquist, 2009). To transiently inhibit Hsp70, we employed a dominant-negative variant of this chaperone (Lagaudrière-Gesbert et al., 2002). We transformed the wild strains with a plasmid encoding this variant and an antibiotic resistance marker. Cells were then allowed to lose the plasmid, restoring normal Hsp70 function. All variants heritably lost the ability to grow on GLY + GlcN after this transient inhibition of Hsp70 activity (Table S2). Although these variants could in principle differ from spontaneous [*GAR*$^+$] in other unknown ways, they have the defining features of this prion and provide resistance to *g*lucose-*a*ssociated *r*epression. For the sake of brevity, we hitherto refer to these variants as [*GAR*$^+$]. We conclude that the variants were due to de novo acquisition of [*GAR*$^+$] and that [*GAR*$^+$] switching rates have been shaped by the diverse ecological niches of the original strains.

[*GAR*$^+$] Occurs Naturally in Wild Strains

Seven of the wild *S. cerevisiae* soil isolates obtained from the laboratory of Fred Dietrich (Diezmann and Dietrich, 2009) (some isolated from Oconeechee Park, VA and some from Stone Mountain Park, GA, USA) behaved as though they already harbored [*GAR*$^+$]. That is, all cells in glucose-grown cultures were immediately able to grow on GLY + GlcN and retained this ability after many hundreds of generations of passage on nonselective media (Figure 2A). This was not true for other *S. cerevisiae* soil isolates in general (nor for other isolates obtained from those same parks or from the Dietrich laboratory). In these strains, as in other wild strains, such variants had to be selected.

We asked whether the unusual ability of these cells to grow on GLY + GlcN was due to the fact that they already contained [*GAR*$^+$]. Indeed, in three of the strains (two from Stone Mountain Park and one from Oconeechee Park), the trait was cured by transiently inhibiting Hsp70 function with the dominant-negative Hsp70 variant (Figure 2B; Table S2). In these same three strains, transient chemical inhibition of Hsp70 had the same long-lasting, heritable effect (Table S2). Moreover, each of these strains had strong repression of *HXT3* mRNA that disappeared after curing

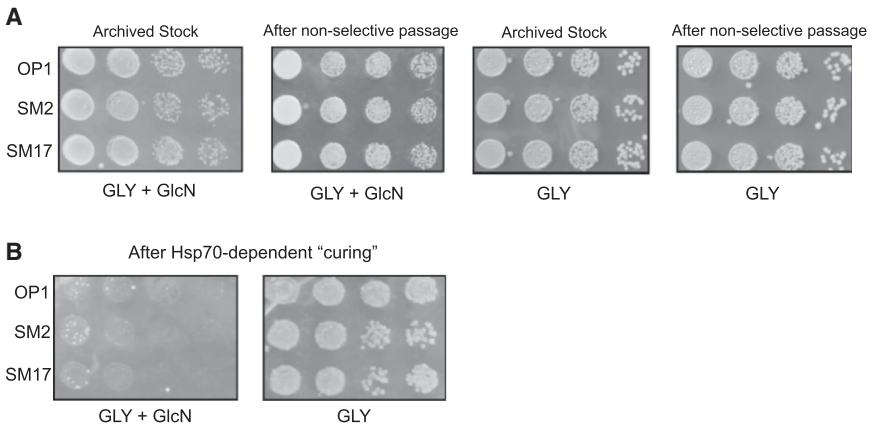


Figure 2. Soil Isolates Are Naturally [*GAR*$^+$]

(A) Three soil isolates grew robustly on GLY + GlcN even after many generations of nonselective propagation.

(B) These same isolates lost this trait after transient reduction in Hsp70 function from a dominant-negative plasmid.

See also Table S2.

with dominant-negative Hsp70 (Table S2). Thus, for at least these three soil isolates, their immediate ability to grow on GLY + GlcN appears to depend on the epigenetic [*GAR*$^+$] element. Whether the other four strains initially acquired the trait via [*GAR*$^+$] (and were subsequently subject to genetic fixation) or whether they acquired it via other means cannot currently be determined. In any case, like the prions [*PSI*$^+$], [*RNQ*$^+$], and [*MOT3*$^+$] (Halfmann et al., 2012), [*GAR*$^+$] is found in wild yeasts.

[*GAR*$^+$]-like Reversal of Glucose Repression Exists in Other Fungi

Next we asked whether protein-based epigenetic elements like [*GAR*$^+$] might exist in other fungi that exhibit robust glucose repression. First we examined two species that diverged from *S. cerevisiae* ~100 million years ago (Langkjaer et al., 2003; Wapinski et al., 2007): *Naumovozyma castellii* and *Candida glabrata*. Glucose repression arose in this lineage prior to their divergence from *S. cerevisiae* (Rozpędowska et al., 2011; Wapinski et al., 2007). Although their glucose repression is not quite as stringent as that of *S. cerevisiae*, it is controlled by a similar genetic network (Rozpędowska et al., 2011).

We grew *N. castellii* and *C. glabrata* in glucose and plated them on GLY plates with and without GlcN. As expected for organisms with robust glucose repression, both species grew well on GLY plates but did not grow well on GLY + GlcN plates (Figure 3A). However, in both, colonies arose on GLY + GlcN plates at a far higher frequency than expected for a trait conferred by mutation ($4.1 \pm 2.8 \times 10^{-3}$ for *N. castellii* and $7.1 \pm 3.6 \times 10^{-4}$ for *C. glabrata*; frequencies determined from six independent biological replicates). Once acquired, the trait was maintained even after passage on nonselective glucose media for hundreds of generations (Figure 3B). Further, the ability of these variants to immediately resume growth on GLY + GlcN was eliminated by transient chemical inhibition of Hsp70 (Figure 3C). We conclude that these species, like *S. cerevisiae*, employ a [*GAR*$^+$]-like switch to circumvent glucose repression.

Prion-Based Reversal of Glucose Repression in a Very Distant Lineage

Next, we turned to *Dekkera bruxellensis*, which diverged from *S. cerevisiae* ~250 million years ago (prior to the appearance of glucose repression in that lineage) (Hellborg and Piškur, 2009). *D. bruxellensis* is employed in the production of Belgian ales and is the only member of its clade known to have evolved glucose repression (Woolfit et al., 2007). It has done so via an entirely different mechanism than *S. cerevisiae*: a rewiring of the regulatory networks that govern respiratory genes (Rozpędowska et al., 2011). As with *S. cerevisiae*, *N. castellii*, and *C. glabrata*, *D. bruxellensis* cells grew well on GLY plates but were unable to grow on GLY + GlcN. Variants that could grow on GLY + GlcN arose at a frequency of ~4 in 10,000 (Figure 3A). Given that *D. bruxellensis* is a diploid organism, this again is a frequency far higher than expected for traits acquired by mutation. As with [*GAR*$^+$] in *S. cerevisiae*, we observed stable strong phenotypes (cells that grew extremely robustly on GLY + GlcN) and weak phenotypes (cells that grew fairly well on GLY + GlcN) (Figure 3B).

Once acquired, the trait was stable through hundreds of mitotic cell divisions. Antibiotic-resistant plasmids have not been used in this organism, limiting options for experimental manipulation. However, the trait was eliminated by transient chemical inhibition of Hsp70 in all ten cases we examined (Figure 3C). After 3 weeks of growth on yeast mold agar medium, we were able to identify asci and isolate 20 spores by microdissection (Kurtzman and Fell, 1998). This allowed us to investigate whether the trait was inherited in a non-Mendelian fashion. We found that all *D. bruxellensis* spores inherited the ability to grow on GLY + GlcN (Figure S2), as is true for non-Mendelian elements such as [*GAR*$^+$] in *S. cerevisiae*. In contrast, DNA sequencing established that polymorphisms segregated randomly, as expected for Mendelian inheritance (Hellborg and Piškur, 2009). Thus, despite having evolved a distinct mechanism for glucose repression, *D. bruxellensis* employs an epigenetic strategy reminiscent of [*GAR*$^+$] to circumvent it.

Turning to comparative genomics, we examined the conservation of key proteins that govern the [*GAR*$^+$] phenotype in *S. cerevisiae* (Pma1, Rgt2, Hxt3, Std1, Mth1) (Brown and Lindquist, 2009). Each of these proteins was highly conserved in *D. bruxellensis*, *N. castellii*, and *C. glabrata*. In contrast, Std1 and Mth1 were not present in *S. pombe* (Figure 4; Tables S3 and S4), which possesses an epigenetic mechanism for reversing glucose repression that does not appear to be prion based (D.F.J. et al., unpublished data). These data strongly suggest that the ability to acquire the [*GAR*$^+$] prion is present in this evolutionarily distant species, and this capacity either has been retained by common descent or has reappeared by convergent or parallel evolution.

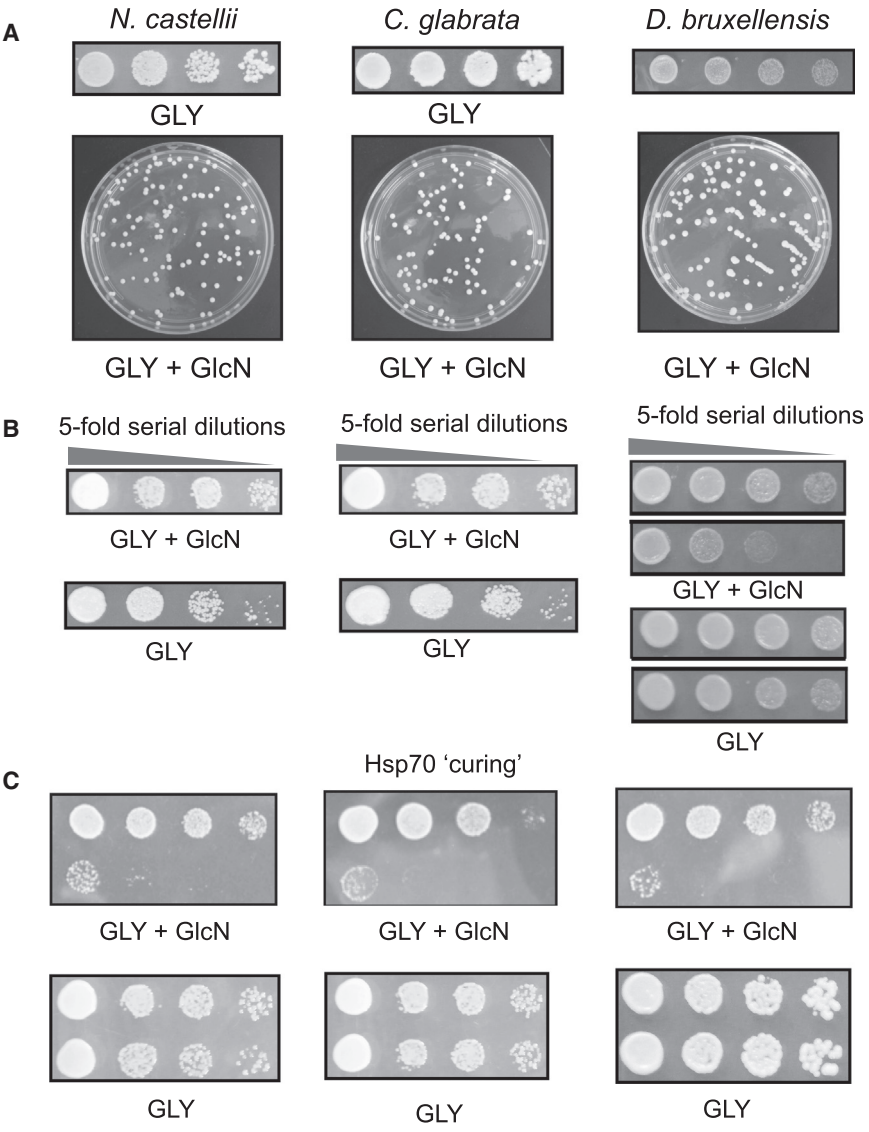


Figure 3. Prion-Based Reversal of Glucose Repression Occurs in Diverse Fungi

Variants of *N. castellii*. *C. glabrata*, and *D. bruxellensis* that (A) could grow on GLY + GlcN medium were stable through (B) multiple passages on nonselective medium but could be eliminated by (C) transient chemical inhibition of Hsp70 (shown here after three passages on GLY plates containing 50 μM myricetin). See also Figure S2.

[*GAR*$^+$] Converts Fungi from Metabolic "Specialists" to Metabolic "Generalists"

In the accompanying paper, we report that when *S. cerevisiae* acquires [*GAR*$^+$], it circumvents that organism's strong specialization for growth on glucose, enabling utilization of a much broader array of carbon sources even when glucose is present. This could confer adaptive benefit in many natural environments where glucose is rare and carbon sources are generally mixed. We therefore examined whether the [*GAR*$^+$]-like epigenetic states in *N. castellii*, *C. glabrata*, and *D. bruxellensis* likewise converted these organisms from metabolic "specialists" to "generalists" (Kassen, 2002) (Figure 5A). To test this, we varied the relative amounts of glucose and multiple other carbon sources (fructose, raffinose, galactose, sucrose, and maltose) in otherwise rich medium. Using these media, we evaluated the growth of both [*GAR*$^+$] and [*gar*$^-$] cells by measuring total biomass yield and doubling time.

The original *N. castellii, C. glabrata*, and *D. bruxellensis* isolates we obtained from genetic stock centers did indeed behave as metabolic specialists: they had high fitness in glucose and low fitness in mixed carbon sources (Figure 5B). In contrast, cells in which the [*GAR*$^+$]-like epigenetic element had appeared acted as generalists. They retained robust growth on glucose but also grew well across a wide range of mixed carbon sources (Figure 5B). Thus, organisms separated by hundreds of millions of years of evolution possess a protein-based epigenetic mechanism that heritably converts cells from metabolic specialists to generalists.

Selection for [*GAR*$^+$] on the Basis of Its Bet-Hedging Properties

In fluctuating environments, the acquisition of phenotypic diversity through a reversible epigenetic mechanism might provide a key adaptive advantage relative to acquisition of such traits by mutation. Mathematical modeling permits quantitative evaluation of this possibility by comparing the spontaneous rates at which a trait arises when it is due to epigenetic switching versus when it is due to genetic mutations (Lancaster and Masel, 2009; Lancaster et al., 2010). Prion-based reversal of glucose repression is dominant, but mutations known to create this trait are recessive (Ball et al., 1976; Kunz and Ball, 1977; Brown and Lindquist, 2009). Thus, comparing diploid and haploid cells for the per-generation rates of colony appearance on GLY + GlcN plates provides a reasonable quantitative assessment of genetic versus epigenetic contributions to this trait (see Extended Experimental Procedures for details).

We used classical Luria-Delbruck fluctuation tests and maximum-likelihood estimations to measure these rates in *S. cerevisiae*, *N. castellii*, *C. glabrata*, and *D. bruxellensis* (Foster, 2006) (Table S5). We then incorporated these values into a previously established mathematical model of bet-hedging (Lancaster and Masel, 2009; Lancaster et al., 2010). Briefly, this model is unique in that it considers both reversible epigenetic variants and irreversible genetic mutations that drive the same phenotype(s). The parameters of the model allow for

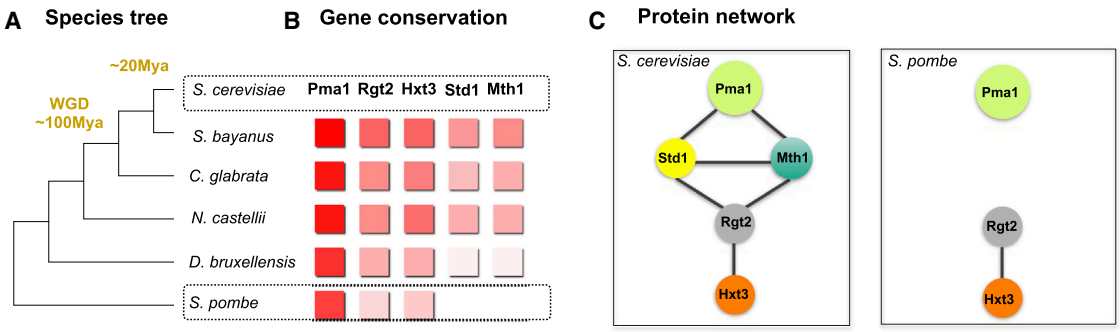


Figure 4. Evolutionary Conservation of [*GAR*$^+$] Signaling Networks across the Fungal Lineage

(A) The species tree for fungi studied.

(B) Presence and absence of homologs for key proteins involved in the [*GAR*$^+$] phenotype. Color indicates the degree of sequence conservation relative to *S. cerevisiae* (see also Tables S3 and S4).

(C) Protein network involved in the [*GAR*$^+$] phenotype in *S. cerevisiae* and predicted consequences of Std1 and Mth1 loss in *S. pombe*.

comparisons of wide ranges of population structure (spatially separated subpopulations), effective population size, and rates of environmental fluctuation. Finally, to make the test even more stringent, we impose an extreme cost on inappropriate switching: cells that do so when it is not advantageous die (Figure 6A). (See the Extended Experimental Procedures for a more detailed explanation of the model and ecological parameter estimation.)

The rates of [*GAR*$^+$] appearance we measured pointed to a strong biological advantage for its maintenance (Figure 6B). This calculation was robust over a wide range of ecological parameters. Strong advantages were also clear for *N. castellii*, *C. glabrata*, and *D. bruxellensis*. Even infrequent rates of environmental change would favor [*GAR*$^+$] over mutational strategies. For example, environmental changes that favored [*GAR*$^+$] only once in every 10,000–1,000,000 generations would be sufficient to explain the retention of this epigenetic element ($N_e\,\Omega \sim 10$, for an effective population size of 10^5 and 10^7, respectively; Extended Experimental Procedures).

To probe the robustness of our inferences, we tested the effects of very low and very high levels of population structure and wide uncertainty in effective population size. Even with these allowances, the calculation for [*GAR*$^+$]'s adaptive value was extremely robust (Figure S3 and Extended Experimental Procedures). Caution is always warranted with mathematical modeling as there may be additional, unknown parameters that act to retain this mechanism. However, our analysis suggests that the phenotypic diversity [*GAR*$^+$] provides by allowing cells to convert between metabolic specialist and generalist lifestyles would alone be sufficient to motivate its evolutionary conservation.

Social Cues Convert [*GAR*$^+$] between a Variable Spontaneous Element and a Concerted Epigenetic Switch

A striking feature of [*GAR*$^+$] in *S. cerevisiae* is its extremely efficient induction by a diffusible factor secreted by bacteria (Jarosz et al., 2014). This social dynamic converts a variable, and spontaneously arising, epigenetic switch affecting the behavior of a few individuals in the population into a concerted switch that determines the metabolic state of most. We asked whether *N. castellii* and *C. glabrata* might also be affected by such interspecies interactions. We screened 45 evolutionarily diverse bacterial species for their ability to induce these fungi to grow on GLY + GlcN (Figure 7; Table S6). Many of the 31 bacterial strains that induced [*GAR*$^+$] in *S. cerevisiae* also induced it in *C. glabrata* and *N. castellii* (Table S6). Once [*GAR*$^+$] appeared in these organisms, it was stable for many hundreds of generations in the absence of bacteria (Figure 7; Table S6).

Six of these bacterial species, but only six, induced *D. bruxellensis* to grow on GLY + GlcN (Table S6). Four of the six strongly induced [*GAR*$^+$] in *S. cerevisiae*, but the other two did not (Table S6). This echoes observations from another cross-kingdom chemical conversation between *Pseudomonas aeruginosa* and *Candida albicans*, which is mediated by a complex ensemble of farnesols (Hogan and Kolter, 2002; Hogan et al., 2004). In that case, too, different bacterial strains produce chemical signals (each sharing a common scaffold) with different induction capacities. Moreover, this result eliminates the trivial possibility that the "inducing bacteria" simply allow growth on GLY + GlcN by metabolizing the GlcN. Rather, the species specificity of the inter-kingdom dialog seems to be tuned to the dynamic selective pressures of life in biologically complex communities. Thus, like [*GAR*$^+$] in *S. cerevisiae*, the [*GAR*$^+$]-like epigenetic elements of evolutionarily distant fungi employ social cues from bacterial organisms to convert an epigenetic element that arises at a low spontaneous rate into a concerted switch.

The Extended Monoculture of Domestication Extinguishes Bacterial Induction of [*GAR*$^+$]

In the accompanying paper, we identified more than 20 gene-deletion mutants in *S. cerevisiae* that abrogated the appearance of [*GAR*$^+$] in response to bacteria but had no effect on the spontaneous appearance of this element. Many of these mutations had no measurable impact on fitness, either in our experiments or in the very extensive analyses of others (Breslow et al., 2008). Clearly, there are multiple routes to extinguishing the acquisition of [*GAR*$^+$] in response to bacterial signals. The question then arises: has this response been retained for the benefits

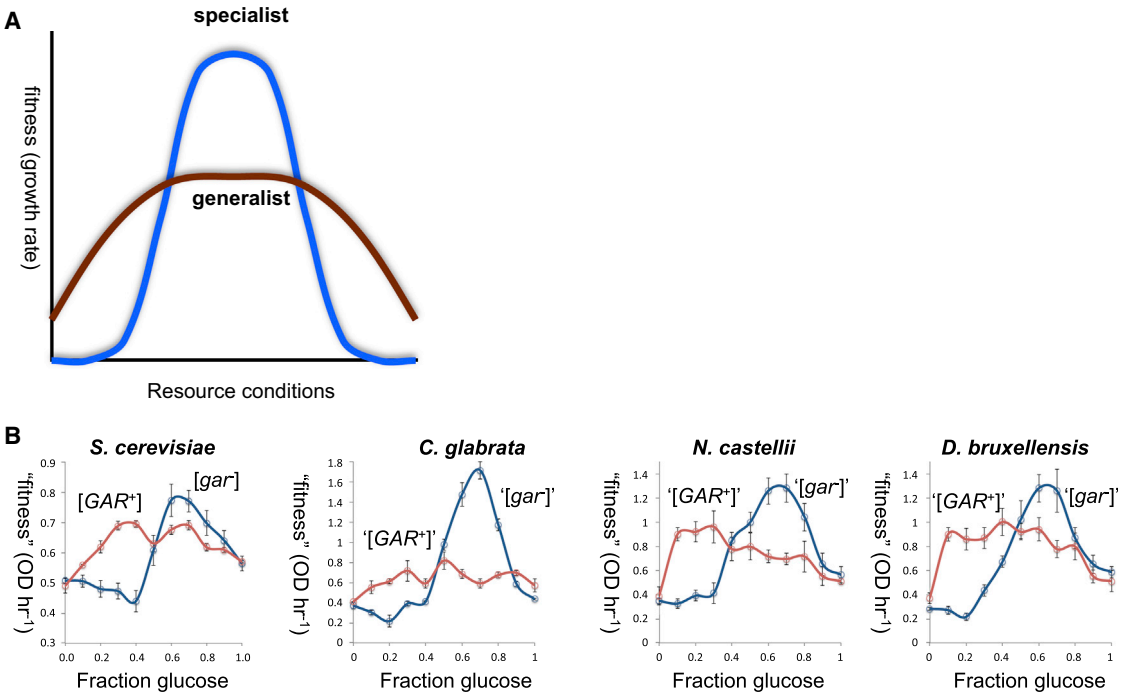


Figure 5. Epigenetic Switches Enable a Generalist Strategy

(A) Schematic of generalist versus specialist strategies for growth and survival.

(B) Normal [*gar*$^-$] cells pursue a specialist strategy (a narrow range of resource conditions with high fitness), whereas [*GAR*$^+$] cells are generalists (fitter across a wider range of conditions). Similar effects are seen with the [*GAR*$^+$]-like states from diverse other fungi. Resource conditions were quantified by the fraction of galactose to total amount of carbon: starting with a carbon source of no glucose and 2% galactose to 2% glucose and no galactose. Fitness was measured as the ratio of final biomass yield to doubling time in exponential phase. Error bars represent the SD from three independent experiments.

that such a communication system might provide in complex social environments?

The monoculture inherent to laboratory domestication provides an opportunity to test this supposition. A hallmark of domestication, from bacteria to nematodes, is the loss of costly mechanisms that are conserved explicitly for the purpose of interspecies social interactions (Velicer et al., 1998; Palková, 2004; Weber et al, 2010; Milward et al, 2011). If fungi have retained this epigenetic switch for the purpose of the adaptive advantages it provides in complex communities, it should be extinguished in at least some laboratory lineages.

Many laboratory yeast strains are ultimately derived from the same initial domestication event. To minimize this potential source of bias, we selected laboratory strains that had independent wild origins. We then compared their abilities to respond to [*GAR*$^+$]-inducing bacterial signals with those of wild strains. Two of the laboratory strains had lost the ability to respond to bacteria altogether, and most of the remaining five were only weakly responsive (Figure 7B; Table S1).

As an additional test, we also compared brewing strains and wine strains. Wine strains and brewing strains are both used by man for the production of alcoholic beverages, but the microbial dynamics of their use are strikingly different. Brewing is characterized by monoculture, employing sterile mashes as a growth substrate. Winemaking, even when fermentations are spiked with defined yeast strains, employs the unsterilized juice or must of crushed grapes, which is replete with bacteria and other fungi (Bisson et al., 2007). Brewing strains were poorly responsive to bacteria. In contrast, wild wine and fruit strains that we tested responded strongly to bacteria in their vicinity (Table S1). It would therefore appear that monoculture, both in the laboratory and in the practice of brewing, is coincident with a repeated dampening of this response to cross-kingdom communication in yeast.

Finally, we asked whether, in the process of domestication, bacterial cells have also lost the capacity to induce the yeast response. We tested four laboratory strains of a Gram-positive bacterium, *Bacillus subtilis*. Three had lost prion-inducing activity (JH642, AG174, and UCD strain 364; Table S6). We also tested six laboratory strains of a Gram-negative bacterium, *Escherichia coli*. Five had lost prion-inducing activity completely (strains AB1157, DH5alpha, BL21, XL1-Blue, and W3110), and in the other, it was weak. Yet all eleven of the independent wild isolates of this species that we tested robustly induced [*GAR*$^+$] (Figure 7B; Table S6). Thus, the ability of bacteria to secrete this prion-inducing factor and the ability of yeast to respond have each been repeatedly lost during domestication. It is of course impossible to discern with absolute certainty whether such loss was caused by or coincident with domestication. Nonetheless, its repeated occurrence suggests that the ability of bacteria to secrete this prion-inducing factor, and the ability of fungi to respond, could have been

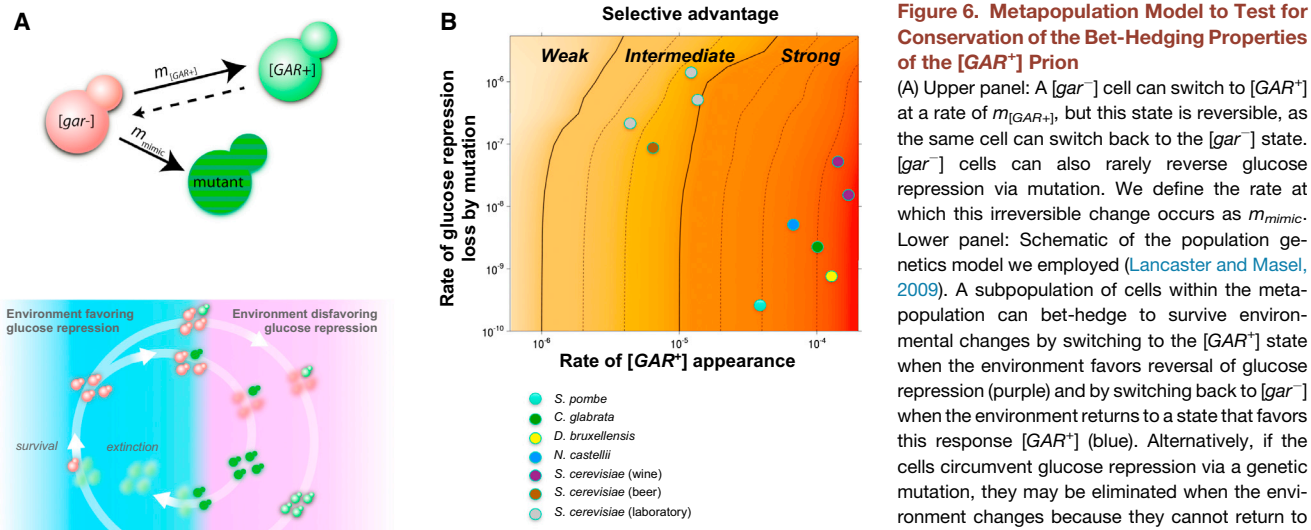


Figure 6. Metapopulation Model to Test for Conservation of the Bet-Hedging Properties of the [*GAR*$^+$] Prion

(A) Upper panel: A [*gar*$^-$] cell can switch to [*GAR*$^+$] at a rate of $m_{[GAR+]}$, but this state is reversible, as the same cell can switch back to the [*gar*$^-$] state. [*gar*$^-$] cells can also rarely reverse glucose repression via mutation. We define the rate at which this irreversible change occurs as m_{mimic}. Lower panel: Schematic of the population genetics model we employed (Lancaster and Masel, 2009). A subpopulation of cells within the metapopulation can bet-hedge to survive environmental changes by switching to the [*GAR*$^+$] state when the environment favors reversal of glucose repression (purple) and by switching back to [*gar*$^-$] when the environment returns to a state that favors this response [*GAR*$^+$] (blue). Alternatively, if the cells circumvent glucose repression via a genetic mutation, they may be eliminated when the environment changes because they cannot return to the [*gar*$^-$] state. (The equal time the subpopulation spends in both kinds of environments is for illustrative purposes only: in the model, environmental changes occur independently and stochastically within each subpopulation.)

(B) Inference of strong selection pressure for [*GAR*$^+$] switching. We define Ω as the rate of environmental change for which the [*GAR*$^+$] phenotype is adaptive. For an effective population size of $N_e = 5 \times 10^6$ (see Extended Experimental Procedures), the contour plot depicts the inferred strength of selection (measured by the product $N_e\,\Omega$) as a function of $m_{[GAR+]}$ and m_{mimic}. For illustrative purposes, we have divided the selection landscape into three regions: weak selection ($1 < N_e\Omega < 5$, colored in cream); moderate selection ($5 < N_e\,\Omega < 50$, colored in orange); and strong selection ($N_e\Omega > 50$, colored in red). Superimposed on the contour plot are the maximum-likelihood estimates for $m_{[GAR+]}$ for each of the *S. cerevisiae* strains and for the other species. Mimic rates appear to be very small; however, we estimated an upper limit to the uncertainty of this parameter of $3.15–7.88 \times 10^{-6}$. The depicted m_{mimic} therefore ranges from the very low (10^{-10}) to this upper limit, and we placed strains equispaced on the vertical axis across this range. See Extended Experimental Procedures and Table S5 for more explanation and details of computations. See also Figure S3 where we validated the robustness of these analyses to a wide range of uncertainty in the parameters.

conserved in nature for the purpose of cross-kingdom social communication.

DISCUSSION

Our findings establish that an epigenetic mechanism that converts yeast from metabolic specialists to metabolic generalists has been broadly conserved in fungi. Further, our work suggests that this conservation may have been driven by the benefits it provides in complex natural environments. [*GAR*$^+$] allows fungi to switch between a metabolic strategy dedicated to fermenting glucose and maximizing ethanol production and a metabolic strategy simultaneously capable of exploiting diverse carbon sources even when glucose is present. The frequency with which the prion appears creates dynamic populations in which some individuals bet-hedge and adopt a different metabolic strategy than the majority. This element, [*GAR*$^+$], has been conserved over at least one hundred million years of evolution (among *S. cerevisiae, N. castellii,* and *C. glabrata*). Moreover, an organism that diverged from *S. cerevisiae* ~300 million years ago and uses a different mechanism for glucose repression, *D. bruxellensis,* also circumvents this repression via a similar epigenetic mechanism. All of these epigenetic elements share several distinguishing features of [*GAR*$^+$]: they arise spontaneously at higher frequencies than expected for mutations, they are dominant, their inheritance critically depends upon the protein chaperone Hsp70, and they are induced by secreted bacterial factors.

The adaptive value of [*GAR*$^+$] is substantial. As a frame of reference, the advantages [*GAR*$^+$] confers to these diverse fungi for growth on complex carbohydrates are quantitatively similar to those that have been measured for many DNA-based genetic variants of *S. cerevisiae*. Indeed, when tested exhaustively over hundreds of different growth conditions, 30% of the gene knockout mutations in this organism produce fitness effects that are smaller than those produced by the loss of [*GAR*$^+$] (Breslow et al., 2008). Whether [*GAR*$^+$] initially evolved as a means to facilitate survival in fluctuating natural environments is, of course, uncertain. However, our mathematical modeling indicates that [*GAR*$^+$] and the [*GAR*$^+$]-like elements of other fungi have been maintained, at least in part, for the adaptive value they confer.

In *S. cerevisiae* at least, the number of individuals that spontaneously place "bets" (with [*GAR*$^+$]) has been tuned to the ecological niche from which the strain is derived. The frequency at which these bets are placed ranges over several orders of magnitude, but this frequency is a stable and reproducible property of that particular strain. Strikingly, [*GAR*$^+$] and the related epigenetic elements we describe here are wired to convert from a spontaneously arising metabolic bet that a small percentage of the population adopts to a concerted, environmentally regulated switch. This drives the majority of cells in the population to heritably change their metabolic program. As described in the accompanying paper, this strategy produces benefits for yeast and bacteria alike.

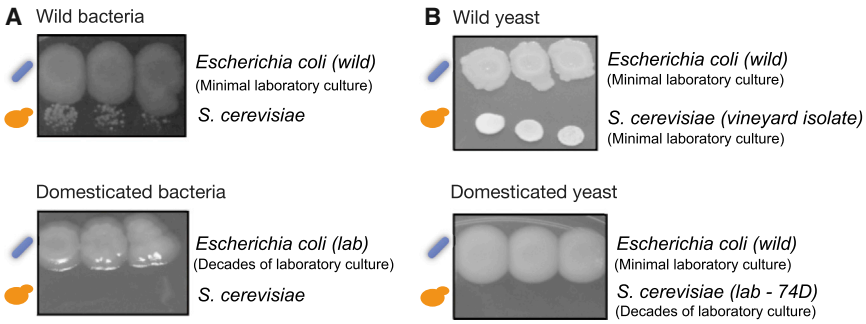


Figure 7. Domestication Extinguishes the Capacity of Bacteria to Secrete a [*GAR*$^+$]-Inducing Signal and the Capacity of Yeast to Perceive It

(A) "Wild" bacteria (e.g., *E. coli* strain MG1655) are better able to induce yeast (*S. cerevisiae* strain W303) to grow on GLY + GlcN medium than domesticated bacteria (e.g., *E. coli* strain W3110). (B) Wild yeast (e.g., *S. cerevisiae* strain UCD2780) are better able to be induced to acquire [*GAR*$^+$] than domesticated yeast strains (e.g., *S. cerevisiae* strain 74D).

See also Table S6.

An evolved prion-based bet-hedging mechanism may have provided a foundation for the subsequent evolution of the [*GAR*$^+$] epigenetic switch in response to secreted bacterial factors. Indeed, emerging evidence suggests that evolution can produce networks that easily interconvert between bet-hedging and switch-based strategies (Beaumont et al., 2009; Levy and Siegal, 2012). It is also possible that the bacterially induced switch evolved first and was then co-opted to serve as a bet-hedging strategy in the absence of prokaryotic competitors. In either case, both our mathematical modeling and our experimental observation that frequencies of bets are tuned to a strain's ecological niche provide strong support for the evolutionary retention of this prion-based bet-hedging strategy. Further suggestive evidence for the adaptive value of this cross-kingdom communication in complex biological communities comes from our observation that during the prolonged monoculture inherent to laboratory and industrial domestication, bacteria have repeatedly lost the ability to "speak" and fungi have repeatedly lost the ability to "listen."

Although no other bet-hedging strategies of this type have yet been described, mechanisms that allow highly adapted metabolic specialists to revert to an ancestral generalist metabolic lifestyle seem inherently appealing. Given how intensely glucose utilization has been studied in yeast, and how robust and highly conserved this mechanism is, it might also seem astonishing that this cross-kingdom communication has not been reported previously. The answer to this puzzle, in part, must lie in our observation that strains cultured extensively in the laboratory have not needed, and have often lost, the capacity for such metabolic versatility.

Studies of man's microbiome are beginning to uncover properties of those populations that profoundly influence human health. We also note that a virtually universal property of human cells during oncogenic transformation is to shift from respiratory to glycolytic metabolism (Lunt and Vander Heiden, 2011). This shift is at least partly regulated by epigenetic mechanisms and involves the integration of complex signaling events between tumor cells and their microenvironment. In this case, the ensuing metabolic versatility is adaptive to that subpopulation of cancer cells but, of course, acts to the great detriment of the whole organism. Controlling this process is an increasing focus of anti-tumor strategies (Lunt and Vander Heiden, 2011). Although yeast prions may seem to operate in a rather distant realm, it seems likely that self-perpetuating epigenetic mechanisms for governing changes in metabolism, such as those we report here, will prove to be commonly deployed in biological systems.

In the past, prudence has rightly dictated that scientific experimentation should generally be conducted on well-characterized strains, in highly defined conditions, in isolation from other organisms. However, in nature, this situation virtually never occurs. Given the detailed levels of understanding we have now achieved with monoculture and defined conditions, the time is ripe to explore another world of biology, that unveiled by wild isolates, natural environments, and community dynamics.

EXPERIMENTAL PROCEDURES

Fungal Strains and Plasmids

All fungi were propagated on standard laboratory media, and GLY + GlcN plates were made as previously described (Ball et al., 1976; Kunz and Ball, 1977; Brown and Lindquist, 2009). *HXT3* levels were determined by RT-PCR using SYBR green quantification relative to an *ACT1* control. Dominant-negative Hsp70 was expressed from a GPD promoter on a plasmid that encoded G418 resistance and was based on the advanced gateway construct pAG42. Loss of this plasmid was accomplished by propagation on nonselective medium (YPD) for 75–100 generations and confirmed by examination of individual colonies for loss of G418 resistance. We also "cured" cells through transient chemical inhibition of Hsp70, using the Hsp70 inhibitors myricetin (50 μM), pifithrin (25 μM), and CE-148 (25 μM; a gift from Dr. Jason Gestwicki). *D. bruxellensis* was sporulated by growing cells on Yeast Mold Agar (Difco) for 3 weeks. After digesting asci with zymolyase, spores were separated by micromanipulation and grown on rich medium (YPD) at 25°C before assessing whether they had retained the ability to grow on GLY + GlcN. *S. pombe* was grown, mated, and sporulated according to standard procedures (Forsburg and Rhind, 2006). Bacterial induction of the ability of fungi to grow on GLY + GlcN medium was measured by plating serial dilutions of each organism in adjacent rows on solid agar plates. Growth of both organisms was measured after 5 days of incubation at 30°C.

Estimating Mimic and [*GAR*$^+$] Appearance Rates

We estimated the rates at which glucose repression is circumvented by reversible epigenetic mechanisms versus genetic mutations by comparing rates from classical Luria-Delbruck fluctuation tests in haploids versus diploids in the W303 strain. Using the observed appearance rates of [*GAR*$^+$]-like phenotypes in haploid and diploid, respectively, and using previous genome-wide estimates of the proportion of mutations that are dominant in yeast, we derived equations to estimate ranges of spontaneous appearance of [*GAR*$^+$] ($m_{[GAR+]}$) and mutation rate (m_{mimic}) for all strains (Table S5) (see Extended Experimental Procedures).

Model for Inferring Strength of Selection

The strength of selection for bet-hedging properties as a function of optimal switching rate ($m_{[GAR+]}$) and mutation rate (m_{mimic}) was computed using the

previously published model of Lancaster and Masel (2009). We set ecological model parameters such as effective population size and gene flow based on previous estimates in *S. cerevisiae* (see Extended Experimental Procedures). By placing our measured estimates of $m_{[GAR+]}$ and m_{mimic} from the fluctuation tests described above, on the surface computed from the model, we inferred the strength of selection for those estimates, assuming that the measured $m_{[GAR+]}$ represents an optimal rate. Our inferences of moderate-to-strong selection were largely robust to different ecological assumptions about effective population sizes and population structure (Figure S3; Extended Experimental Procedures).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.07.024.

AUTHOR CONTRIBUTIONS

The authors have made the following declarations about their contributions. D.F.J., A.K.L., J.C.S.B., and S.L. conceived and designed the experiments. D.F.J. and J.C.S.B. performed the experiments. D.F.J. and A.K.L. performed data analysis. A.K.L. performed mathematical modeling and bioinformatics. Co-first authors D.F.J. and A.K.L. and senior author S.L. wrote the paper.

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