Published in final edited form as:

Nat Ecol Evol. 2019 November ; 3(11): 1539–1551. doi:10.1038/s41559-019-0993-0.

Single Nucleotide Mapping of Trait Space Reveals Pareto Fronts that Constrain Adaptation

Yuping Li,

Departments of Biology, Stanford University, Stanford, California 94305, USA

Dmitri A. Petrov,

Departments of Biology, Stanford University, Stanford, California 94305, USA

Gavin Sherlock

Departments of Genetics, Stanford University, Stanford, California 94305, USA

Abstract

Tradeoffs constrain the improvement of performance of multiple traits simultaneously. Such tradeoffs define Pareto fronts, which represent a set of optimal individuals that cannot be improved in any one trait without reducing performance in another. Surprisingly, experimental evolution often yields genotypes with improved performance in all measured traits, perhaps indicating an absence of tradeoffs at least in the short-term. Here we densely sample adaptive mutations in *S. cerevisiae* to ask whether first-step adaptive mutations result in tradeoffs during the growth cycle. We isolated thousands of adaptive clones evolved under carefully chosen conditions and quantified their performances in each part of the growth cycle. We too find that some first-step adaptive mutations can improve all traits to a modest extent. However, our dense sampling allowed us to identify tradeoffs and establish the existence of Pareto fronts between fermentation and respiration, and between respiration and stationary phases. Moreover, we establish that no single mutation in the ancestral genome can circumvent the detected tradeoffs. Finally, we sequenced hundreds of these adaptive clones, revealing novel targets of adaptation and defining the genetic basis of the identified tradeoffs.

Correspondence to Dmitri A. Petrov (dpetrov@stanford.edu) or Gavin Sherlock (gsherloc@stanford.edu).

Author Contributions

Conceptualization: Y.L., G.S., and D.A.P. Methodology: Y.L.. Formal Analysis: Y.L.. Investigation: Y.L.. Writing – Original Draft: Y.L.. Writing – Review & Editing: Y.L., D.A.P., and G.S.. Supervision: D.A.P., and G.S..

Competing Interests

The authors declare no competing interests.

Data Availability

All sequencing data are deposited in Short Read Archive under Bioproject ID PRJNA515761. The rest of data is available in the main text or the supplementary tables. Figure 2 and 3 have associated raw data. All strains are readily available from authors upon request.

Code Availability

All code used in this manuscript are deposited in GitHub. The source code for computing these fitness estimates can be found at <https://github.com/barcoding-bfa/fitness-assay-python>. The source code for variants calling and annotation can be found at <https://github.com/liyuping927/DNAscope-variants-calling>.

Introduction

That gain must ultimately be associated with some cost is a fundamental premise in fields spanning economics, engineering, and biology. Biology in particular has a rich tradition of both alluding to and attempting to define tradeoffs: here tradeoffs imply that a part of trait space is not accessible by evolution, such that, within a defined period of time, a lineage cannot evolve improved performance of two or more traits simultaneously above some threshold. Such evolutionary tradeoffs have been suggested by various biological phenomena - for instance, organisms with high fecundity tend to have a short lifespan¹⁻³ and organisms with large eggs tend to lay fewer of them^{4,5}.

Despite the plethora of such examples of negative correlations between specific traits, such correlations alone are insufficient to demonstrate the existence of tradeoffs. Indeed, many alternative explanations exist. For instance, consider an environment in which only one trait is under selection while a second is not. Over evolutionary time, performance in the first trait is likely to increase while performance of the second is likely to decrease due to the accumulation of damaging mutations in the absence of purifying selection^{6,7}. At the same time, a reciprocal relationship may be observed in an alternative environment if the second trait is subject to selection and the first one is not. This will lead to a negative correlation between performances of the two traits. However, it is entirely possible that mutations that improve both traits do exist, but they are not particularly common and not particularly advantageous in either of the environments. Additional explanations, such as sexual selection driving some traits to seemingly suboptimal states⁸, or current selective pressures not corresponding to the way natural selection acted in the past might also lead to negative correlations among traits in the absence of tradeoffs. In short, negative correlation in performance between two traits is expected in the presence of tradeoffs but in and of itself is not sufficiently strong evidence for the existence of tradeoffs.

Consider an organism with two traits under selection (Fig. 1a): its trait-fitness space is two-dimensional, with each axis representing performance for one of the traits. If a tradeoff exists between the two traits, for every biologically possible value of trait 1, the best value for trait 2 performance will be constrained by trait 1, generating a Pareto optimality front (or Pareto front)⁹. Such a Pareto front not only represents the set of optimal trait combinations, but also separates the “accessible” from the “inaccessible” trait space. For individuals on the Pareto front (green dots in Fig. 1a), the existence of tradeoffs can be demonstrated straightforwardly: increasing the performance for one trait will inevitably decrease performance for another. By contrast, individuals behind the Pareto front (the black dot in Fig. 1a) are able to improve performance in both traits simultaneously. It is generally assumed that organisms should be located on or near a “long-term” Pareto front as they are products of very long term evolution^{1,2,5,9-12}. Surprisingly, results from experimental evolution often demonstrate the improvement of multiple traits simultaneously, suggesting that at least for the conditions and traits tested, the ancestor does not lie on a Pareto front¹³⁻¹⁹. However, it is important to appreciate that it is possible for an individual to be on a higher dimensional Pareto front, defined by multiple traits, but when measuring only a subset of the traits, the organism will appear to be behind the front (Fig. 1b). In this case,

improvement in performance in the subset of traits must come at the cost of performance in the additional, unmeasured, traits that contribute to the higher dimensional front.

The Pareto front is typically thought of as being defined by physical, structural, or physiological constraints and is usually considered to be static. However, the Pareto front may also be defined by genetic constraints, such that the space above the front might be locally inaccessible in the short-term due to the rarity of specific genetic changes required to reach that region of trait space. For example, if the “inaccessible” part of trait space requires the system to move through a fitness valley the system might remain at the ‘Pareto front’ at least in the short term. The transition into the locally inaccessible part of the space would then be seen as a true evolutionary innovation that shifts the Pareto front to a new location. The Pareto front is thus defined both by the timescale of evolution and the physiological or structural relationships among the traits and therefore may be dynamic over time.

To explore whether even the first step of adaptation can reveal evolutionary constraints in the form of Pareto fronts, one needs to sample a large number of adaptive mutants selected for multiple traits under a range of conditions and then precisely measure their performance along each trait axis (Fig. 1c,d). Pareto fronts, if present, can then be inferred by an absence of mutants able to maximize both traits simultaneously (the large red dot in Fig. 1a,c). If the first step mutations can reach the short-term Pareto optimality front and if the density of sampling is such that any adaptive single-step mutant that would land beyond the defined front would have been detected with high likelihood, then a short-term Pareto front will have been demonstrated.

Here we set out to investigate the existence of Pareto fronts among multiple traits, by evolving barcoded yeast populations under a number of carefully chosen conditions, selecting for improved performance in different phases of the yeast growth cycle, including fermentation, respiration, and stationary phases. We isolated ~500 independent adaptive clones most of which carry a single beneficial mutation. We found that a number of adaptive clones improved all three measured performances to a modest extent without apparent tradeoffs, indicating that the ancestor cannot be located on a Pareto front for the measured traits. However, no adaptive clones were able to maximize performance in some pairs of traits. We were able to delineate apparent Pareto fronts between fermentation and respiration as well as between respiration and stationary phases, but not between fermentation and stationary phase performances in our short-term evolution experiments. Importantly, due to a large number of sampled and tested clones we could assert that no single point mutation in the genome of the founding yeast strain in our experiment can improve the performance substantially beyond either of the two defined Pareto fronts. Finally, by sequencing hundreds of adaptive clones, we identified the genetic basis underlying the identified tradeoffs and revealed novel targets of adaptation.

Results

Experimental System and Isolation of Evolved Clones

When yeast cells grow in conditions with a fermentable carbon source, such as glucose used in this study, they go through a sequence of growth phases: (i) lag phase, where cells

acclimate to the medium, with no cell division; (ii) fermentation, where cells divide exponentially by converting glucose into ethanol; (iii) respiration, where glucose is exhausted and cells divide slowly by consuming the ethanol produced during fermentation; and (iv) stationary/starvation phase, where cells cease growth because readily-available carbon has been depleted from the medium (Fig. 2a).

To isolate adaptive clones with improved performances in fermentation, respiration, and/or stationary phase (or combinations thereof) we propagated barcoded haploid yeast populations under four serial transfer conditions having differing cycle lengths: 1) 1-day (referred to as Evo1D below) including 4h lag, 16h fermentation, and 4h respiration; 2) 2-day (Evo2D, conducted in Levy, Blundell *et al*²⁰) including additional 24h respiration; 3) 5-day (Evo5D) including a further 12h respiration and 60h stationary phase; and 4) alternating 1-day and 5-day transfer (Evo1/5D) (Fig. 2a). Note that even though the same growth medium was used in the above conditions, we refer to them as different conditions as the differences in their transfer periods generate profoundly different selective pressures. We used barcode trajectories to determine that cell cultures in cycle 11 contained a high proportion of diverse adaptive clones. Furthermore, our previous analysis indicated that at this time point most adaptive clones would contain only a single adaptive mutation²⁰. Subsequent sequencing of individual clones (Venkataram, Dunn *et al*²¹ and see below) confirmed this supposition.

We isolated clones from cycle 11 for subsequent analysis. Specifically, from Evo1D, Evo2D, Evo5D, and Evo1/5D we isolated respectively 120, 3048 (isolated in Venkataram, Dunn *et al*²¹), 157, and 384 distinct evolved clones carrying unique barcodes. We previously found that ~50% of clones isolated from Evo2D had self-diploidized during the course of evolution²¹ and were beneficial across all fitness measurement conditions²². We therefore assayed the ploidy of newly isolated clones, and observed 43%, 45%, and 14% diploids among clones isolated from Evo1D, Evo5D, and Evo1/5D, respectively.

We measured the fitness of all isolated clones in 1-day (Fit1D), 2-day (Fit2D), 3-day (Fit3D) and 5-day (Fit5D) serial transfer conditions (Fig. 2b, clones from Evo2D were measured in Li, Venkataram *et al*²²) using the method developed in Venkataram, Dunn *et al*²¹. For each clone, we therefore have its fitness in the “home” condition (except for Evo1/5D clones), as well as the “away” conditions. Note that one condition (Fit3D) was not used as an evolutionary condition but instead was important for evaluating stationary phase performance. Below we use these values to investigate patterns of local adaptation and to estimate performance of each clone in fermentation, respiration, and stationary phases. Using the fitness and ploidy measurements, we identified 66, 144, 58, and 132 adaptive haploids and 4, 40, 57, and 6 high-fitness diploids (assumed to have additional beneficial mutations besides diploidy) from Evo1D, Evo2D, Evo5D, and Evo1/5D, respectively. We refer to these adaptive haploids and high-fitness diploids collectively as adaptive clones.

Local Adaptation Results from Performance Differences in Different Growth Phases

We observed a large range of fitness both in the “home” and “away” environments (Fig. 2c). For example, the fitness of all adaptive clones varied from -0.35 to +2.2 per growth cycle in Fit5D, suggesting multiple adaptive strategies and targets of adaptation among these clones.

While only 4.5% of the adaptive clones are maladaptive in any away condition, we do find that in general, adaptive clones exhibit evidence of local adaptation. Specifically, for each fitness remeasurement condition, both the average and the highest fitness of clones evolved in the home condition (indicated by arrows) are greater than those of clones evolved in the away conditions. Nonetheless, under a given fitness measurement condition, not all “home” clones are more fit than all “away” clones.

We further used our combined fitness data to determine the *performance* of individual clones in three of the phases in the growth cycle: fermentation, respiration, and stationary phase (Fig. 2d). Here, as cells spend different amounts of time in each growth phase, we define performance as the increase in fitness, *per hour*, for a given growth phase; our previous study demonstrated that the overall fitness scales linearly with the amount of time spent in each of the growth phases²². The slope of the relationship between the relative fitness of a clone and the length of a particular growth phase (measured as fitness change per hour) can thus be used as a measure of clone performance in that phase. For instance, as the clones spend 24 extra hours in respiration during every cycle when growing under Fit2D compared to Fit1D we can calculate respiration performance by subtracting relative fitness of each clone in Fit1D from that in Fit2D and then dividing by 24 hours: $(\text{Fit2D} - \text{Fit1D})/24$. Similarly, we calculated the fermentation $((\text{Fit1D} - 4 \times \text{respiration performance})/16$, as cells go through 4h respiration and 16h fermentation in 1-day condition) and stationary performances $((\text{Fit5D} - \text{Fit3D})/48)$ (Methods section 6).

We compared these three performances for clones evolved in all four conditions. Overall, while clones from each condition often revealed specific and consistent patterns of apparent tradeoffs, the tradeoffs observed were not necessarily shared across all conditions (Fig. 2d,e). For example, we previously found that most adaptive clones from Evo2D have improved performance in both fermentation and respiration, but decreased performance in stationary phase²². By contrast, adaptive clones from Evo1D have improved performance in fermentation, yet decreased performance in respiration and nearly unchanged performance in stationary phase. Most adaptive clones from Evo5D exhibit yet a different pattern -- improved performance in both fermentation and stationary phases but their performance in respiration on average is largely unchanged. Finally, adaptive clones from Evo1/5D have improved fermentation and stationary phase performance and generally decreased respiration performance. Overall, we found adaptive clones that improved every pair of fermentation, respiration, and stationary phase performances, as well as some that showed improved performance across all three (indicated by arrows in Fig. 2e), suggesting that the ancestor is behind any potential Pareto front for these three performances.

The Genetic Basis of Adaptation and Tradeoffs

We determined the genetic basis of adaptation by genome-wide sequencing of 47, 67, and 85 adaptive clones from Evo1D, Evo5D, and Evo1/5D respectively. Putative adaptive mutations were successfully identified in 35 (74%), 66 (98%), and 81 (95%) of these clones. The identity of 125 adaptive mutants from Evo2D was determined previously^{21,22}. Many genes or pathways were recurrently mutated in our adaptive clones – in such cases we can be confident that these mutations are indeed adaptive. Specifically, out of the 182 adaptive

clones in which we identified putative adaptive mutations, 118 (~65%) harbor mutations in genes/pathways hit in multiple clones (Supplementary Table 3). Furthermore, 79 of them harbor mutations in genes/pathways independently hit five or more times (Table 1).

In general, within each evolutionary condition, beneficial mutations were limited to a small number of genes that serve similar biological functions. At the same time, across evolutionary conditions, beneficial mutations tend to differ in their genetic bases (Table 1). For instance, we previously reported that the majority of adaptive mutants for Evo2D upregulated the *RAS/PKA* and *TOR/Sch9* nutrient sensing pathways²¹, but we rarely recovered adaptive mutations in these pathways from the other evolutionary conditions. By contrast, loss of function in *SXMI* (also known as *KAP108*, which encodes a β -karyopherin^{23,24}) was the prevalent cause of adaptation in Evo1D. Karyopherins mediate transport through the nuclear pore. *Sxm1* functions as an importin, and in an *shm1* strain, the localization of Tpk2 (one of the catalytic subunits of protein kinase A (PKA)) to the nucleus in response to stress is abrogated²⁵, and its chromatin association is abolished. Thus, in an adaptive strain from Evo1D carrying a loss of function mutation in *SXMI* it is possible that Ras/PKA activity is diminished, potentially explaining the loss of fitness in respiration experienced by these mutants. While *SXMI* mutants were also observed in Evo5D, they were not the predominant mutant class. Instead, a wide variety of mutations were observed among Evo5D adaptive clones, including (i) 11 duplications of chromosome 11 (*Chr11Dup*), (ii) 10 independent loss of function mutations in *FPK1*, and (iii) 9 mutations in three components of the high-osmolarity glycerol (*HOG*) response pathway: *SSK1*, *SSK2*, and *HOG1*. Given that Evo5D contains a long period of starvation, observation of *Chr11* aneuploidy is consistent with previous findings that aneuploidies can improve survival under extremely stressful conditions^{26–28}, although the underlying mechanism is unknown. *FPK1* (a flippase activator) has been previously shown to increase viability in stationary phase²⁹, which we experimentally confirmed (Supplementary Table 4). The genetic bases of adaptation among Evo1/5D clones were similar to those for Evo5D clones, with mutations in *SXMI* and *FPK1* as well as duplication of *Chr11*.

Next, we examined the relationship between the identified genetic basis of adaptation and the resulting increases/decreases in performance (Fig. 3a–c). As stated above, in this study “performance” represents fitness change per hour in a particular growth phase rather than measurements of physiological traits (e.g. growth rate) as it is commonly used. Due to population dynamics, the genetic outcome of each evolutionary condition is biased towards the observation of mutations in genes with a large target size and high fitness. Even though we have sampled a large number of clones from multiple conditions, mutations that arise too infrequently or are insufficiently adaptive do not end up contributing to adaptation in the culture and thus are not sampled. For instance, the *SXMI* mutants, predominant in Evo1D, have among the highest observed fermentation performances, at >6% per hour (giving >96% fitness advantage over the ancestor over the full 16-hour period of fermentation in our conditions). This likely explains why nutrient-sensing *RAS/PKA* and *TOR/Sch9* pathway mutants, which have lower fermentation performance than the *SXMI* mutants, were not observed in Evo1D. However, the high fermentation performances of *SXMI* mutants come at a cost of reduced respiration performance (negative 2–3% per hour). This likely explains their near absence in Evo2D given that the Evo2D condition contains a long period of

respiration. This allows the *RAS/PKA* and *TOR/Sch9* mutants to dominate the Evo2D conditions. Similarly, the most prevalent Evo2D *RAS/PKA* nutrient-sensing pathway mutants with the highest respiration performance tradeoff strongly in stationary phase²², explaining why they were not observed in Evo5D. Finally, clones that are common in Evo5D, which contains all phases of the growth cycle, are the least likely to show decreased performance in any phases of the growth cycle. Indeed, Evo5D specific mutations, such as *Chr11* duplication and *SSK1* mutation, show no obvious tradeoffs, but rather modest improvements in one or more performances (Supplementary Fig. 1). Interestingly, Evo5D clones also include *SXMI* mutants that show increased performance only in fermentation with decreased performance in respiration and little change in stationary phase. In this case, their strong improvement in fermentation and lack of tradeoff in stationary phase appears to compensate enough for their reduced fitness in respiration.

In summary, adaptation under these conditions is idiosyncratic yet predictable: the genetic basis of adaptation under a particular evolutionary condition tends to target a narrow, recurrent and thus *a posteriori* predictable set of genes. However, these gene targets are not shared across all environments, meaning that adaptation across conditions often relies on entirely different genetic pathways. This idiosyncratic nature explains the specific patterns of performances across conditions (Fig. 2d,e). While we do detect clones that increase all performances, clones that perform best in any one growth phase tend to tradeoff in performance in some other growth phase(s). This hints at the existence of evolutionary constraints, preventing the emergence of adaptive clones that simultaneously *maximize* performance in all growth phases.

Identification of Evolutionary Constraints and Delineation of Pareto Fronts

We observed an absence of clones near the upper limits of either both fermentation and respiration performances, or both respiration and stationary performances (the large red dot in Fig. 3a,d and 3b,e). Thus, there is at least the appearance of an empty space in the upper right corner, where these pairs of performances would be maximized. We used the convex hull algorithm to delineate potential Pareto fronts that separate the short-term evolutionarily-accessible space from the empty, putatively short-term inaccessible space above the front (grey curves in Fig. 3).

We first tested whether, given the marginal distributions of trait performances, the size of the empty space at the top right of those plots is surprisingly large (Methods section 10). Under a null hypothesis of independence of performances, the observation of such large empty spaces is indeed unexpected ($P < 1\text{E-}3$ for fermentation and respiration phases and $P = 4\text{E-}3$ for respiration and stationary phases, respectively; Fig. 3d,e and Supplementary Fig. 2). By contrast, the size of the empty space between fermentation and stationary performances is not unexpected (Fig. 3f and Supplementary Fig. 2; $P = 0.76$).

The Mutational Target Size of the Optimal Types is Smaller Than A Single Nucleotide

To further explore the absence of clones beyond the putative Pareto fronts, we determined the target size for possible single-step mutations that would give rise to the maximum performances for fermentation and respiration, or respiration and stationary phase (marked

by the large red dot in Fig. 3a,d and 3b,e). Mutants that could maximize two traits simultaneously would be more fit than the observed mutants at least in some evolutionary conditions; thus, based on this increased fitness, such mutants, should they arise at a similar rate as the observed mutants, should be sampled frequently in those conditions. For example, mutants that improve fermentation and respiration simultaneously beyond the putative front should have a higher fitness than most of sampled clones in Evo2D (Supplementary Fig. 3), as clones in this condition experience only fermentation and respiration. Likewise, clones that improve respiration and stationary phase beyond the putative front should have a high fitness in Evo5D (Supplementary Fig. 3), given that the majority of clones with high respiration or stationary performance have a positive fermentation performance as well. The fact that we didn't observe any clones beyond the putative fronts suggests that the genomic mutational target size towards such extremely fit mutants located beyond the putative Pareto fronts must be smaller than that for the observed mutants. Furthermore, we demonstrated that our evolution experiments were not mutation limited. Indeed, given the observed mutation rate of $\sim 3\text{E-}10$ per base pair (bp) per generation (1bp is mutated into a specific nucleotide A, T, C, or G), the effective population size of $\sim 6\text{E}8$ and the number of generations before sampling (~ 80), each base pair is expected to have been mutated into one specific nucleotide ~ 15 times by the time of our sampling.

Next, we used a mathematical model to quantitatively assess the probability of sampling a single-step mutation with a given selection coefficient s (Methods section 11). Several factors determine the probability of sampling such a single-step mutation: the rate at which a mutation occurs, the probability of such a mutation surviving random drift and establishing in the population ($\sim$ proportional to s), and the exponential division rate after the mutation establishes (its cell number roughly reaches $e(s*t)$, with t generations between establishment and sampling). With mutations entering the population at a fixed rate, the more fit a mutant is (the larger s is), the more likely the mutant establishes in the population, the faster the mutant divides and eventually the higher frequency the mutant reaches by the sampling time.

First, consider a gene with the same target size for adaptive mutations as *IRAI* (mutations in which were observed 39 times after sampling at cycle 11 of Evo2D^{21,22}), but whose mutation results in a fitness benefit at the hypothetical optimal type, with maximal fermentation and respiration (the red dot in Fig. 3a,d). Such a hypothetical mutant would have a fitness of ~ 2.56 per cycle in Evo2D, compared to ~ 1.64 per cycle for *IRAI*-nonsense mutations. If such a hypothetical gene exists, we would expect to observe mutations in this gene $\sim 25,000$ times more frequently than we observed mutations in *IRAI* in Evo2D ($(e(2.56*11)/e(1.64*11))$). Thus, it is exceptionally unlikely that such a gene with a similar target size to *IRAI* does exist. Furthermore, if the target size for such a gene is just a single base pair, our mathematical model suggests that we would expect to see such a mutation between 84 and 99 percent of the time in our evolution experiments (Methods section 11). Thus, we believe it is unlikely that there is even a single site in the genome of the ancestral strain that can be mutated to provide such a high fitness.

Similarly, the hypothetical optimal type which maximizes the respiration and stationary phase performances would have a fitness benefit of ~ 2.98 per cycle in Evo5D (represented by the red dot in Fig. 3b,e) (assuming a fermentation performance of zero). If a single site

(1bp) can be mutated to this hypothetical optimal type, we would expect to sample such a mutant between 88 and 98 percent of the time in Evo5D experiments. Thus, there is likely no single-step mutation in the ancestral yeast genome that can simultaneously maximize either both fermentation and respiration, or both respiration and stationary performances to their highest observed levels.

Discussion

A Large Number of Diversely Selected Adaptive Clones Is Needed to Delineate Pareto Fronts

Despite the fact that tradeoffs have been widely assumed in studies of evolution, it is extremely challenging to formally establish the existence of tradeoffs. Here, by sampling a large number of adaptive clones from a range of evolutionary conditions, and measuring their performance in three different traits, we were able to demonstrate the existence of Pareto fronts between fermentation and respiration, and between respiration and stationary phase performances. Furthermore, we were able to show that the ancestor must be behind these fronts, because for both pairs of traits there were clones that were able to improve performance in both traits simultaneously; indeed, some clones were able to improve performance in all three traits.

If the ancestor was on a front delineated by two traits, characterization of the front using experimental evolution would be straightforward, because no adaptive clones could improve both traits simultaneously – indeed, by definition, improvement of performance in one trait would lead to a loss of performance in the other. However, because the ancestor lies behind the fronts we identified, only by mapping a very large number of adaptive clones whose performances span the trait space could we map the Pareto fronts. By randomly subsampling our data, we estimated that between ~100–300 independent adaptive mutants are required to detect the Pareto fronts in our experiment (Methods section 10). Furthermore, given that clones isolated from a particular evolutionary condition, e.g. Evo1D, tend to occupy a specific part of the trait space, clones from Evo1D, Evo2D, and Evo5D together were required to detect the Pareto fronts.

Finally, having such a large number of adaptive clones enabled us to show that for both of the identified Pareto fronts there is no single mutation that can occur in the genome of the ancestral strain that would enable the strain to maximize performance in both traits. These fronts therefore constrain the evolutionarily accessible space over short timescales.

No Observed Pareto Front between Fermentation and Stationary Phase

We were unable to identify a Pareto front between fermentation and stationary phase performances, suggesting either an absence of tradeoffs between these two traits or that single-step mutations provide insufficient performance improvement to reach a hypothetical Pareto front between these two traits. However, this may also be due to experimental limitations: specifically, clones selected under Evo5D experienced both fermentation and respiration prior to stationary phase. Thus, it is entirely possible that the maximum stationary phase performance is larger than we observed, if clones with such a large

stationary phase performance tradeoff strongly in fermentation or respiration. A longer stationary phase, e.g. a 10-day serial transfer, may help select for such mutants and define a Pareto front between fermentation and stationary phase performances should one exist. Additionally, evolution in a non-fermentable carbon source followed by a long stationary phase may also enable selection of clones with high stationary phase performance that tradeoff strongly in fermentation.

The Shape of Pareto Fronts and Nature of Tradeoffs

Levin (1962)³⁰ suggested that the geometry of Pareto fronts will affect an organism's evolvability, and whether generalists or specialists will tend to evolve. For instance, a convex-shaped front allows for better evolvability and produces different optimal types based on the particular evolutionary condition, allowing for local adaptation (Fig. 4a). By contrast, a concave-shaped front leads to less evolvability, because regardless of the importance of performance in each trait, one of the two most specialized types will always be the most fit (Fig. 4b).

Previous studies have used, for example, ecological data in phytoplankton³¹, interactions between phage and *E. coli*³², and synthetic, *E. coli* based systems³³ to investigate the geometry of Pareto fronts, and in one case, it has been shown that an evolving ancestor is likely on a Pareto front¹². However, no study has yet quantitatively defined a Pareto front or characterized its geometry in evolving populations where the ancestor lies behind the front, which is the case in most experimental evolutions. Here we identified not one, but two convex-shaped fronts for two independent tradeoffs under well-controlled selection pressures in our short-term evolution experiments. There are two important points to consider. First, we note that despite evolving under different transfer regimes that subjected cell to distinct selective pressures, each regime used the same growth medium with a defined glucose concentration. It will be important to determine whether even subtle variations to this growth medium would change the selective pressures and in doing so, would alter or even eliminate the observed Pareto fronts. Second, it is possible that the shape of the Pareto front itself may change over the timescales of evolution^{34,35} and the way in which it might change will be informative about whether the observed front is due solely to a genetic constraint, or instead whether there is an underlying intrinsic physiological constraint.

Over longer-term evolution, the space that is inaccessible in the short term may become populated, and the shape change to become a rectangle (Fig. 4c). This would imply there is no physiological constraint between the two traits and the observed Pareto front is purely due to a genetic constraint – that is, no clones with single mutations are able to occupy the seemingly inaccessible space, yet clones with multiple mutations can. Alternatively, the front may either stay in place (Fig. 4d), or move forward but retain the same shape (Fig. 4e), always defining an inaccessible space. This scenario would suggest intrinsic physiological constraints that no single individual could maximize performances in both traits simultaneously. A final possibility is that longer-term evolution may change the shape of the front from being convex to being concave (Fig. 4f) such that individuals with extreme performance in one or the other trait are the most fit depending upon the exact condition in which they are evolved.

The behavior of clones containing multiple adaptive mutations should provide some insights. We observed three clones carrying two adaptive mutations each in genes specific to different evolutionary conditions. These clones harbor mutations in *SXMI* and *HOG1*, *SXMI* and *SSK1*, and *SXMI* and *CYR1*, respectively. We observed that each of these double mutants is no closer to the front than the corresponding single mutants (Supplementary Fig. 4), suggesting the front itself might be moderately stable. However, clearly both long-term evolution and further evolution of already adaptive clones under various conditions are needed to test this.

Future Prospects

Despite much focus on the study of tradeoffs in ecology and evolution, rigorous demonstration of tradeoffs has proven surprisingly difficult^{15,36}. Furthermore, even when tradeoffs have been demonstrated, the underlying causes typically remain elusive -- the genetic bases of adaptation and tradeoffs identified here provide additional potential targets for further investigation of whether the detected tradeoffs are caused by intrinsic physiological constraints. Here we have shown that it is possible to use barcoding and experimental evolution across a range of conditions to isolate a large enough number of adaptive mutants that together can map the shape of the evolutionary accessible trait space in short-term evolution, from which tradeoffs can be inferred. Our approach is generic and can be used to study tradeoffs between multiple traits including ecologically relevant traits such as the ability to sporulate or undergo mating and can be performed with different founding strains and species. Such studies hold promise in helping us to understand the shape of tradeoffs among multiple traits both in pairs and in higher dimensions.

Methods

1 Founding Populations and Experimental Evolution

1.1 Barcoded yeast populations—Barcoded yeast populations were constructed as in Levy, Blundell *et al.*²⁰. However, where Levy, Blundell *et al.*²⁰ used only one landing pad yeast strain (SHA185), we used multiple landing pad yeast strains, with each landing pad strain carrying a unique, condition-specific barcode. Next, a high-complexity plasmid library was introduced into each landing pad strain through transformation; correct integrants were selected for uracil prototrophy²⁰. These transformants contain both a low-complexity *condition-specific* barcode and a high-complexity *lineage tracking* barcode. Three barcoded yeast populations with distinct condition-specific barcodes were constructed. Each barcoded yeast population includes around half a million unique transformants. These three barcoded yeast population were evolved under Evo1D, Evo5D and Evo1/5D, respectively.

1.2 Yeast growth media and growth cycle—All cultures were grown in 100 mL of M3 medium²⁰ (5x Delft medium with 4% ammonium sulfate and 1.5% dextrose) in 500 mL Delong flasks (Bellco) at 30°C and 223 RPM. This culture condition is referred to as our standard culture condition.

Yeast cells go through different growth phases when growing under a glucose-limited condition, including the lag phase, fermentation, respiration and the stationary phase. Based

on our previous study²², with 5E7 cells inoculated into our standard culture condition, cells experience lag phase for ~4 hours, fermentation for ~16 hours, respiration for ~40 hours and stationary phase thereafter²². Wild-type cultures reach a saturation cell number ~1.7E8/mL and thus the final population size is ~1.7E10. We note that these parameters can change somewhat under different growth conditions. For example, cells experience a longer lag phase under Evo5D since cells entered into stationary phase in the previous growth cycle. Such variations are not taken into account in our analyses. Further, these parameters are variable across different genotypes. For instance, the tested *IRA1-nonsense* mutation saturated around 1.1E8/mL after 2-day growth²².

1.3 Serial-batch transfers during evolution—The barcoded yeast populations were evolved by serial batch culture in M3 medium. Under Evo1D, bottlenecks were performed by adding 7E7 cells of the culture from the previous growth cycle to fresh media; cells were grown for 1 day/24 hours between each bottleneck. Cells were highly viable (close to 100%) after 1 day of growth. Cell counts were performed at each bottleneck to estimate the generation time. Cultures reached ~9E7 cells/mL with a final population size ~9E9. Thus, cells went through ~7 generations during each transfer in Evo1D ($\log_2(9E9/7E7) = 7$ generations). Populations were evolved for up to 25 growth cycles (~175 generations).

Under Evo5D, bottlenecks were performed every 5 day/120 hours. Cultures reach ~1.25E8 cells/mL with a final population size ~1.25E10. Cells' viability decreased during the stationary phase. To avoid strong bottleneck drift, the cells' viability was estimated at each bottleneck and 7E7 viable cells were inoculated to the next serial transfer. Viability was estimated by counting colony forming units (CFUs) on YPD plates and then dividing CFUs by the number of cells plated. Cells went through ~7.5 generations during each transfer in Evo5D. Cells were evolved up to 16 cycles (~120 generations).

Under Evo1/5D, cells were bottlenecked every 1 day and 5 days, alternating. 7E7 viable cells were inoculated at each bottleneck. Similarly, cells went through ~7 generations during 1-day transfer and ~7.5 generations during 5-day transfer. Cells were evolved up to 23 cycles by alternating between 1 day and 5 day transfers (~166 generations).

Two replicates were conducted under each evolutionary condition. At the end of each transfer, 2mL cell culture of evolution were mixed with 1ml 40% glycerol, aliquoted into two Eppendorf tubes and stored at -80°C. The rest of the cell culture (>95mL, besides a small amount used to inoculate the next cycle), was centrifuged, then the cell pellet was resuspended in 5mL sorbitol solution (0.9M sorbitol, 0.1M Tris-HCL pH 7.5, 0.1M EDTA pH 8.0), and then aliquoted into 2mL Eppendorf tubes and stored at -20°C for genomic extraction.

In addition, Evo2D evolution was conducted in Levy, Blundell *et al.*²⁰, where bottleneck was performed by adding 400μL (~5E7 cells) to fresh media. Cells went through ~8 generations per cycle.

2 Tracking Lineage Dynamics During Evolution

2.1 Library preparation and sequencing—To track lineages' frequency changes over the course of evolution, cells collected at the end of every three transfers under Evo1D, Evo5D and Evo1/5D were used for genomic DNA extraction. Genome extraction and PCR amplification of the barcode region were conducted as in Venkataram, Dunn *et al*²¹. Amplicons were sequenced on Illumina HiSeq 4000 (2X101 paired end). Data for Evo2D can be found in Levy, Blundell *et al*²⁰. A perfect sequencing read should follow this DNA sequence:

```
NNNNNNNNXXXXTTAATATGGACTAAAGGAGGCTTTTGTCTGACGGATCCGATAT
CGGTACC (+26bp lineage Barcode+) ATAACTTCGTATAATGTATGCTATACGAAGTTAT
(+26bp condition barcode+)
GGTACCGATATCAGATCTAAGCTTGAATTCGATxrefXXXXXXXXNNNNNN
```

The Ns in the sequence are random nucleotides and are used as UMIs in the downstream analysis to remove skew in the counts caused by PCR jack-potting²⁰. The Xs correspond to multiplexing tags, which allow different samples to be distinguished when loaded on the same sequencing flow cell²⁰.

2.2 Barcode counts—Sequencing reads, with 17bp (including the UMIs and Multiplexing tags) on both the 5' and the 3' end removed, were first aligned to the reference barcode region using Bowtie2. Based on the alignment, both condition and lineage barcodes were extracted. Next, the UMIs, Multiplexing tags and barcodes were re-associated for each pair of reads. Reads were split into different files based on their Multiplexing tags, representing which sample the reads came from. Lastly, reads from the same sample were clustered using Bartender³⁷ with Hamming distance 2 and seed length 8. The final output includes both barcode sequences and counts of each lineage. Each lineage is represented by a unique combination of condition and lineage barcodes. Reads with the same combination of UMI, Multiplexing tags and barcodes were counted as one read as they are likely caused by PCR jack-potting. An extra round of clustering was conducted using barcode combinations identified by Bartender to further cluster barcodes with ≤ 2 Hamming distance. On average, each sample has ~1.3 million high-quality reads. For each evolution replicate, lineages with fewer than 10 counts across all time points and lineages present at fewer than three sequenced time points were filtered out. Barcode counts over the course of evolution can be found in Supplementary Table 1.

3 Isolated Clones from Evolution

3.1 Isolate evolved clones from evolutionary conditions—Based on population dynamics, yeast clones were isolated from frozen samples of Evo1D, Evo5D and Evo1/5D at cycle 11. ~50μL of frozen stock (containing ~3.5E6 cells) was diluted into 500μL PBS plus 1μL propidium iodide and used for flow cytometry sorting. Single cells were sorted into 96-well plates with 100μL YPD media in each well. 16 plates of cells (~1,500 cells) were sorted for each evolutionary condition with 8 plates from each replicate (48 plates in total sorted from Evo1D, Evo5D and Evo1/5D). Sorted cells were grown at 30°C for three days without shaking and reached saturation by day three. ~5μL of saturated cell culture were removed from each well and inoculated into a different 96-well plate with 100μL fresh YPD media in

each well. These replicated plates were grown at 30°C for two days without shaking to reach saturation and used for following ploidy and barcode identification. The rest of the saturated cell culture was mixed with 100µL 50% glycerol and stored at –80°C.

3.2 The ploidy test—A high throughput ploidy test was developed using the drug benomyl²¹. The saturated cultures from above 48 replicated 96-well plates were mixed and pinned onto YPD+20µg/ml benomyl (in DMSO) rectangular agar plates using a multi-pronged pinner, grown at 25°C for 48 hours, and then imaged. Under these conditions, diploid growth is strongly inhibited by benomyl but haploid growth is less affected.

3.3 DNA barcode identification by Metagrid—20µL of saturated cell culture were removed from each well of the replicated plates into 96-well PCR plates. Cells were lysed by incubating at 95°C for 15 mins. 5µL of lysed cell culture were used to PCR amplify the DNA barcode region. To reduce the cost of Sanger sequencing ~4,600 clones, a Metagrid approach was developed. 72 forward and 64 reverse primers were synthesized with each primer carrying an 8bp unique multiplexing tag (Ns in the primer sequence). Each PCR well was coded by a unique combination of forward and reverse multiplexing tags. Two-step PCR amplification was conducted here with the first step using multiplexed primers and the second step using standard Illumina paired-end ligation primers (PE1 and PE2). PCR products from all 48 plates were pooled together and sequenced using Illumina NextSeq (2X150 paired end).

Forward Primer of the First Step

PCR: ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNTTAATATGGA
CTAAAGGAGGCTTTT

Reverse Primer of the First Step

PCR: CTCGGCATTCTGCTGAACCGCTCTTCCGATCTNNNNNNNNNTCGAATTCAA
GCTTAGATCTGATA

Regular expressions that match DNA sequences flanking multiplexing tags of primers and clones' barcodes were used to extract multiplexing tags and barcodes. A map between clones' DNA barcodes and their physical positions on the 96-well plates was constructed based on multiplexing tags. The number of clones carrying unique barcodes is lower than the number of clones isolated due to the lack of growth in a small number of wells and more importantly, the high frequency of a few adaptive clones -- a large number of isolated clones carrying the same barcodes. 124, 166, and 397 unique barcodes were identified from Evo1D, Evo5D and Evo1/5D respectively.

4. High-throughput Fitness Measurements

4.1 Pool of clones for fitness measurements—Isolated clones with unique barcodes were pooled together for high-throughput fitness assays. Note that some barcodes were present in both haploid and diploid clones; for these barcodes, only haploid clones were pooled for fitness assays. Clones with unique barcodes were hand-picked from previous frozen stock and re-arrayed onto a set of 96 deep-well plates with 700µL YPD medium in each well. Cells were grown at 30°C for 2 days to reach saturation without

shaking. 500µL of 50% glycerol were added into each well using a multichannel pipette. 1mL of the mixture from each well was pooled together into a container. The pooled cell culture was mixed well and aliquoted into 2mL Eppendorf tubes, which were stored at 80°C for future fitness measurements.

4.2 Fitness measurement conditions—We measured clones' fitness under four conditions, 1-day, 2-day, 3-day and 5-day serial batch culture conditions (Fit1D, Fit2D, Fit3D and Fit5D). These four conditions are the same except the length of the growth cycle. Out of which, 1-day, 2-day and 5-day serial batch culture conditions are the same as Evo1D, Evo2D and Evo5D evolutionary conditions. Note that fitness of Evo2D clones were measured in Li, Venkataram *et al*²². Clones isolated from Evo1D, Evo5D, and Evo1/5D were measured in this study and analyzed in this section. Note that all data reported in this section can be found in Supplementary Table 2.

4.3 Preculture—A tube of pooled cell culture was removed from –80°C and thawed at room temperature. 1mL of pooled cell culture was inoculated into 15ml fresh M3 media contained in a 500ml Delong flask and grown at 30°C shaking at 223 RPM overnight for cell propagation. 400µL of overnight cell culture were inoculated into 100mL fresh M3 media (X4) and precultured at the standard condition.

An ancestral clone carrying a restriction site in the barcode region was previously constructed and used to compete with evolved clones for fitness measurements²¹. The ancestor clone was streaked out from a freezer stock onto M3 agar plates and grown for 2 days until colonies were visible. A single colony was inoculated into 3mL of M3 medium and grown for 48 hours (30°C roller drum). After saturation, 400µL were used to inoculate pre-cultures (100mL M3 media in 500mL Delong flasks, 223 RPM 30°C). Like the pooled cell culture, four pre-cultures were prepared.

The pooled preculture and the ancestor preculture were acclimated for the same cycle length as that of the fitness measurement condition. For instance, precultures were grown for one day before being mixed and assayed under the 1-day fitness measurement condition.

4.4 High-throughput competition—Fitness assays were conducted by mixing the pooled preculture with the ancestor preculture in a 1:9 ratio (time 0) and growing this mixed culture for four successive growth cycles (time points 1, 2, 3 and 4). In Fit1D, at the end of each cycle, 450µL cell culture was inoculated into 100ml fresh media to start the next cycle. In Fit2D, Fit3D and Fit5D, at the end of each cycle, 400µL cell culture was inoculated. Cells reached a lower cell number after 1-day growth compared to after 2-day, 3-day and 5-day growth. Thus, we inoculated a larger volume to ensure that the number of cells inoculated were consistent across fitness measurement conditions. In addition, we inoculated 450µL to ensure that the cell culture was close to saturation after 1-day growth and thus yielded enough cells for downstream analyses. Cells were collected at time 0, and at the end of each of the four growth cycles. Cell pellet from each sample was resuspend in 5ml sorbitol solution (0.9M sorbitol, 0.1M Tris-HCL pH 7.5, 0.1M EDTA pH 8.0), aliquoted into 2mL Eppendorf tubes and stored at –20°C. Three replicates were performed under each fitness

assay condition. Genome extraction, barcode region amplification and Illumina sequencing were conducted for each sample.

4.5 Library preparation of high-throughput fitness assays—Genomic DNA was extracted with following steps: 1) remove collected cells from -20°C and thaw at room temperature; 2) spin down cells and wash them with water; 3) resuspend cells in 400 μL buffer (0.9 M Sorbitol, 50mM Na Phosphate pH 7.5, 240 $\mu\text{g}/\text{mL}$ zymolase, 14mM β -mercaptoethanol) and incubate at 37°C for 30 minutes; 4) add 40 μL 0.5M EDTA and vortex; 5) Add 40 μL 10% SDS and vortex; 6) add 56 μL 20mg/mL proteinase K (Life Technologies 25530–015), vortex very briefly and incubate at 65°C for 30 minutes; 7) incubate samples on ice for 5 minutes; 8) add 200 μL 5M potassium acetate, shake and incubate on ice for 30 minutes; 9) spin for 10 minutes, transfer supernatant to a new tube with 750 μL isopropanol and rest on ice for 5 minutes; 10) spin down for 10 minutes and wash twice with 70% ethanol; 11) resuspend in 50 μL 10mM Tris pH 7.5 -- leave on bench overnight if pellet is not resuspended fully; and 12) add 0.5 μL 20 mg/mL RNase A and incubate at 65°C for 30 minutes.

Two-step PCR was used to amplify the barcode region. See Venkataram, Dunn *et al*²¹ for primer details. The first step PCR was conducted using 6 μg genomic DNA and separated into six 50 μL reactions.

OneTaq 2X Mix 150 μL

Forward Primer 5uM 3 μL

Reverse Primer 5uM 3 μL

Template 6 μg

MgCl₂ 50mM 12 μL

H₂O add up to 300 μL

PCR program for the first step PCR:

1X 94°C 10 minutes

3X 94°C 3 minutes

55°C 1 minute

68°C 1 minute

1X 68°C 1 minute

4°C inf

We then performed PCR cleanups following the QIAquick PCR purification protocol (6 PCR reactions pooled into one column) and elute into 30 μL H₂O.

The second step PCR was completed in one reaction using Herculase II fusion DNA polymerase (Agilent 600677):

Buffer 10 μ L

dNTP 100 μ M 0.5 μ L

Herculase 0.5 μ L

PE1 10 μ M 1.25 μ L

PE2 10 μ M 1.25 μ L

Template 30 μ L

H₂O 6.5 μ L

PCR program for the second step PCR:

1x 98°C 2 minutes

20x 98°C 10 seconds

69°C 20 seconds

72°C 30 seconds

1x 72°C 1 minute

4°C inf

4.6 Fitness estimates—The number of DNA barcodes was tracked by Illumina sequencing (Illumina NextSeq), which was then used to estimate lineages' frequencies in the population as a whole, as previously described^{21,22}. In this study, times 0, 1, 2, and 3 were used for fitness estimates under Fit1D, Fit2D and Fit3D. Time 0, 1, 2, 3, and 4 were used for fitness estimates under Fit5D. The source code for computing these fitness estimates can be found at <https://github.com/barcoding-bfa/fitness-assay-python>. First, we input barcode counts to the script. After the 1st run, the script output barcodes that were likely to be neutral. During the 2nd run, we input both the barcode counts and a list of neutral barcodes estimated from the 1st run. Fitness estimates from the 2nd run were used for further analysis. Final fitness estimates were calculated by inverse variance weighting of estimates from all three replicates. Barcodes identified in these fitness assays were mapped back to barcodes identified in Metagrid. Thus, for each unique barcode, we know the fitness values under all fitness measurement conditions, its physical position in a 96-well plate in frozen stock, and the associated ploidy. Note that the physical position of each barcode is required for picking clones for genome-wide sequencing. In sum, 661 unique barcodes had confidently called ploidy and were successfully identified from our fitness assays. Out of the 661 unique barcodes, 644 of them had high-quality fitness values under every fitness assay condition and were used for further analysis.

5 Classification of Clones

The 644 lineages with high-quality fitness measurements were classified into four groups, based on their ploidy and fitness: neutral haploids, adaptive haploids, diploids presumed to have no additional adaptive mutations (“pure” diploids), and diploids with additional adaptive mutations (high fitness diploids; see Li, Venkataram *et al.*²² for details). Briefly, adaptive haploids were defined as lineages that had low probability ($p < 1e-3$) of having log fitness of 0 or less in at least two conditions; high fitness diploids were defined as diploid lineages with additional adaptive mutations that had low probability ($p < 1e-3$) of having fitness less than the mean fitness of the diploid class in at least one condition. The mean fitness of the diploid population was calculated using inverse variance weighting. In total, 254 adaptive haploids, 66 high fitness diploids, 218 neutral haploids and 106 pure diploids were characterized among clones isolated from Evo1D, Evo5D, and Evo1/5D. See Li, Venkataram *et al.*²² for classification of Evo2D clones.

6 Quantification of Performances in Growth Phases

Fitness change per hour was used as the measurement of lineages’ performances in different growth phases. Respiration performance per hour was calculated by subtracting Fit1D measurements from Fit2D measurements and dividing by 24 hours (because compared to Fit1D, clones experienced 24 hours of extra respiration in Fit2D, while keeping the lag and fermentation phases roughly the same). Fit1D almost directly measures the performance in fermentation but did contain ~4h respiration per transfer. Therefore, using above estimates of respiration performance per hour, we subtracted expected fitness change in the extra 4 hours of respiration from Fit1D measurements. We then divided the difference by 16 hours of fermentation to estimate fermentation performance per hour. Lastly, stationary phase performance per hour was calculated by subtracting Fit3D from Fit5D measurements and dividing the difference by 48 hours of stationary phase. The same quantification was performed for Evo2D clones, using their fitness measured under 1-day, 2-day, 3-day and 5-day serial transfer conditions²². See Li, Venkataram *et al.*²² for statistical details.

7 Genome-wide Sequencing and Variant Calling

7.1 Genome-wide sequencing library preparation—Clones selected for sequencing were grown in 500μL YPD on 96 deep-well plates for two days at 30°C without shaking. 400μL of cell culture were collected from each well for DNA extraction. Genomic DNA was prepared using Invitrogen PureLink Pro 96 Genomic DNA Kit (Catalog no. K1821–04A) in a 96-well format. Libraries were constructed and multiplexed using Nextera technology with the protocol of Kryazhimskiy, Rice *et al.*⁸. Samples were sequenced with 2×150 Illumina Next-seq paired end sequencing technology. 179 adaptive haploids, 20 high fitness diploids, 8 neutral haploids and 9 pure diploids were sequenced with an average coverage >50 for both haploids and diploids. Note that only clones isolated from Evo1D, Evo5D, and Evo1/5D are sequenced and analyzed in this study and reported in this section. See Venkataram, Dunn *et al.*²¹ and Li, Venkataram *et al.*²² for details about Evo2D clones.

7.2 FASTQ processing—The source code for variants calling and annotation (section 7.2–7.6) can be found at <https://github.com/liyuping927/DNAscope-variants-calling>.

For each sample, we received two fastq files, one for each read of the paired end sequencing (“fastqR1” and “fastqR2”). Using cutadapt version 1.16³⁹, we trimmed the first 10bp of each read (-u 10), low-quality ends (-q 30) and any adapter sequences (-a). After trimming, sequences with a length shorter than 12bp (--minimum-length 12) were discarded. Reads were mapped using bwa⁴⁰ to *S. cerevisiae* S288C reference genome R64-1-1 (https://downloads.yeastgenome.org/sequence/S288C_reference/genome_releases/) and sorted using Sentieon Genomic Tools Version 201711.02⁴¹. Note that bwa used here is the default version incorporated in Sentieon Genomic Tools. Duplicates were removed using the sorted BAM file. Local realignment around indels was performed using the deduped BAM file. Lastly, base quality score recalibration was performed using the realigned BAM file.

7.3 SNP and small Indel variant calling—SNP and small indels variants were called by the DNAscope algorithm (Sentieon Genomic Software) using the realigned BAM file and the output table of the base quality score recalibration. The parameter ploidy is assigned as 1 for haploids and as 2 for diploids.

7.4 Structural variant calling—First, DNAscope algorithm detected the break-end variant type (BND). The parameter ploidy was assigned as 1 for haploids and as 2 for diploids. Second, SVSolver algorithm processed BND and output structural variants to a VCF file.

7.5 Copy number variant (CNV) calling—First, recalibrated BAM files were created using previously generated aligned BAM files and quality score calibration tables. Second, the Panel of normal (PON) was created using recalibrated BAM files. By changing the window size of the bed file, the window size used to call CNVs was varied. Window sizes (200bp and 10kb) were applied in this study. The PON files were created only with the sequencing of haploids. Third, for each strain, the CNVs were called using the strain’s recalibrated BAM file, the bed file and the PON file.

7.6 Variant annotation—vcf files from SNP and small indel variants calling and structural variants calling were further annotated using snpEff⁴² (<http://snpeff.sourceforge.net/download.html>). For variants in coding regions, SNPSift was used to extract the first annotation of each variant, which is the annotation with the largest effect. For variants in non-coding regions, the nearest gene of each variant was extracted. Thus, the non-coding variants were annotated as either the upstream or downstream of the nearest genes.

7.7 Filtering SNPs, small indels and structural variants—First, mitochondrial variants were discarded. Second, background variants, present in all strains, were removed. Variants present in >10 clones, which are isolated from more than one evolutionary condition, were also considered as background variants and discarded. Third, any variants in genes *FLO1* and *FLO9* were filtered out due to poor alignment in both genomic regions. Fourth, variants with a quality score smaller than 200 were filtered out. Note that if a variant was present in multiple clones, the alignment of this variant was manually checked regardless of its quality score and a decision was made based on all clones carrying this variant. Thus, a variant with a quality score <200 may not be filtered out if the same variant

contained in other clones was proven to be authentic. Similarly, a variant with a quality score >200 may be filtered out if the same variant was proven to be bogus in other clones. Only 6 out of ~500 variants had quality scores <200. Lastly, by manually checking BAM files after alignment, variants within repetitive regions and regions with a poor alignment were filtered out. In addition, if a variant is present in multiple clones and these clones carry a same condition barcode, this variant is likely to be pre-existing mutations introduced during the barcoded population construction. These pre-existing mutations can be causative mutations and thus are kept in our analysis.

7.8 Filtering copy number variants (CNVs)—Our DNA extraction and sequencing protocols resulted in an unevenly distributed and relatively low coverage of short chromosomes, including chromosomes I, III and VI. Thus, CNVs associated with these three chromosomes were not considered. For both haploids and diploids, CNVs generated with a 10kb window size were used to identify large duplications and deletions, combined with visual inspection of coverage plots. These variants were further confirmed by CNVs generated with a 200bp window size.

8 Detailed Information of Mutations

179 adaptive haploids, 20 high fitness diploids, 8 neutral haploids and 9 pure diploids from Evo1D, Evo5D, and Evo1/5D were sequenced in this study, with 163 adaptive haploids, 19 high fitness diploids, 8 neutral haploids, and 7 pure diploids have variants identified. All detailed information of variants can be found in Supplementary Table 3.

Pre-existing mutations were identified as 1) mutations in the same gene that are the exact same alleles, and 2) clones carrying identical mutations in the same gene with have the same condition barcodes. Thus, these pre-existing mutations were likely introduced during the construction of barcoded yeast populations. All 8 sequenced neutral haploids and 1 out of 9 sequenced pure diploids harbor chromosome IX duplication, suggesting that chromosome IX duplication is not an adaptive event. Since lineages with chromosome IX duplications have different condition barcodes, it is likely that chromosome IX duplication independently happened during the population construction. In this work, chromosome IX duplications are treated as pre-existing mutations. Note that even though the same pre-existing mutation appear in multiple clones, it is regarded as one independent adaptive event. The pre-existing mutations are not necessarily non-adaptive. For instance, despite pre-existing *SSK1* mutations, *SSK1* mutation also independently happened during evolution and thus is very likely to be adaptive. In addition, *LSM2*, *NUT2*, *TFB3*, *tL(GAG)G* and *VOA1* genes harbor both pre-existing mutations and mutations independently happened during evolution.

Lineages carrying mutations in *SSK1*, *SSK2* or *HOG1* gene are high-osmolarity glycerol (HOG) pathway mutants. Lineages carrying mutations in Ras/PKA pathway genes or TOR/Sch9 pathway genes are referred to as nutrient response pathway mutants in this work: *RAS2*, *GPB1*, *GPB2*, *PDE2*, *IRA1*, *CYR1*, *TFS1* and *YAK1* gene are involved in Ras/PKA signaling pathway; *SCH9*, *TOR1*, *KOG1* and *MDS3* are involved in TOR/Sch9 signaling pathway.

Recurrent mutations within genes or pathways are highly unlikely under neutral evolution and are a hallmark of adaptive mutations. Genes or pathways that were independently mutated five times or more were reported in Table 1 in the main text, including the duplication of chromosome XI that appeared more than five times. The full list of genes that were independently hit more than once can be found in Supplementary Table 3. None of these multi-hit genes/pathways were mutated in neutral haploids and pure diploids sequenced in this study. By contrast, these multi-hit genes/pathways were mutated in 118 out of 182 adaptive clones with identified variants (~65%). In addition, 79 out of 182 adaptive clones (~43%) harbor mutations in genes/pathways that were independently hit more than five times.

Three lineages carry mutations in coding regions of two genes listed in Table 1 in the main text, offering an opportunity to study epistasis among these beneficial mutations. Specifically, these three lineages carry mutations in coding regions of *SXMI* and *SSK1*, *SXMI* and *HOG1* and *SXMI* and *CYRI*, respectively (Supplementary Fig. 4). These three lineages are not colored in Fig. 3a–c in the main text. In addition, diploids with chromosome XI duplication and clones with *SSK2* mutations from Evo2D are not colored in Fig. 3a–c in the main text. If a mutant harbors more than one mutation, as long as one and only one of the mutations is located in genes listed in Table 1, it is classified as a mutant carrying mutations in genes listed in Table 1 (Supplementary Fig. 1).

9 Identification and Fitting of Pareto Optimality Fronts

The fermentation, respiration and stationary performances of adaptive haploids and high fitness diploids from Evo1D, Evo2D, Evo5D, and Evo1/5D were used for the following analysis. The “chull” function in R was used to identify the smallest set of points (the convex set) that when connected, enclosed the rest of points. By connecting the two points with the largest value on x or y, a line was formed. Out of the convex set, points either on or above this line were used to depict the Pareto front. 6, 5 and 3 points were identified to depict the Pareto front between fermentation and respiration performance, between respiration and stationary phase performance and between fermentation and stationary phase performance, respectively.

With known points on Pareto fronts, regression was used, looking for a curve that fit the front. The polynomial fit was evaluated using the Bayesian information criterion (BIC) and the adjusted R^2 , where lower BIC and higher R^2 are considered a better fit. Both criteria include a penalty term for the number of parameters in the model to avoid overfitting. Specifically, first to third degree polynomials were applied to the front between fermentation and respiration performance. We found the values of BIC –27, –40, and –43 and adjusted R^2 of 0.64, 0.96 and 0.97 for 1st, 2nd and 3rd degree polynomial fits, respectively. The 1st degree polynomial fit was rejected due to its poor BIC and adjusted R^2 . Because the 3rd degree polynomial fit provides only a marginally better fit than the 2nd degree polynomial fit, we chose to go with the simpler model. Specifically, the 2nd degree polynomial fit used is $\text{Res} = 0.047 + 0.746 \cdot \text{Fer} - 20.306 \cdot \text{Fer}^2$. Similarly, first to third degree polynomials were applied to the front between respiration and stationary phase performance. BIC –33, –66 and –64 and adjusted R^2 0.81, 1 and 1 were observed for 1st, 2nd and 3rd degree polynomial

fit, respectively. Both BIC and adjusted R^2 supports that the 2nd degree polynomial fit offers the best fit. Specifically, the 2nd degree polynomial fit $\text{Res} = 0.040 - 0.997 \cdot \text{Sta} - 45.650 \cdot \text{Sta}^2$ was used. The second derivatives of both curves are negative, indicating that both curves are convex. Due to the small number of points on the front (including 3 points) between fermentation and stationary phase performance, we were unable to compare the first degree polynomial fit to the second degree polynomial fit.

10 The Inaccessible Space is Not Generated by Chance

We tested whether the observed Pareto fronts and evolutionary inaccessible spaces were generated by random chance. For each pair of performances, the evolutionary inaccessible space was defined as the empty space between the Pareto front (identified by the convex hull algorithm) and the upper limits of both performances. The null hypothesis assumes that a pair of performances are independent from each other.

The error of the fitness estimate is roughly Gaussian (see supplementary information in Venkataram, Dunn, *et al.*, 2016, Cell). Thus, for each fitness measurement, we resampled the fitness from a normal distribution with the estimated mean and standard deviation (resampling with error). Performances were recalculated from the resampled fitness. We then sampled the tested performances independently with replacement for 504 times (the total number of adaptive clones from all four evolutionary conditions; resample with permutation) from the recalculated performances and calculated the amount of empty space under the null hypothesis.

We repeated the above test for 1,000 times and calculated, under the null hypothesis, the chance of observing an empty space equal to or larger than the empty space generated by our real data (Supplementary Fig. 2). For fermentation and respiration performances, none of the permutations had an empty space as large as in the real data ($p < 0.001$). For respiration and stationary performances, four of the permutations had an empty space as large as in the real data ($p = 0.004$). By contrast, for fermentation and stationary performances, when we sampled the marginal distributions, the empty space we observed in the real data sits within the distribution of the empty spaces generated by the permutations. 761 of the permutations had an empty space larger than observed in the real data ($p = 0.76$).

We then calculated the minimum number of clones that is needed to define the inaccessible space. By random sampling current adaptive clones, we concluded that between 80 and 300 clones are required to confidently ($P \leq 0.01$) define the inaccessible space between fermentation and respiration performance, and between respiration and stationary phase performance, respectively.

11 Mathematical Model to Calculate the Probability of not Sampling Optimal Types

11.1 Equations—By knowing the selection coefficient (s per generation) of a particular adaptive event, the bottleneck population size (N_b), the mutation rate (μ) and the effective variance of cell division per generation ($2c$), we can estimate the density distribution of establishment time (τ) of this adaptive event (equation 7 in Supplementary Information of Levy, Blundell *et al.*²⁰). This density distribution represents for a given time point the

probability that an adaptive event occurs and rises to c/s cells to avoid drift (see Levy, Blundell *et al*²⁰ for details).

$$p(\tau) d\tau = s/\Gamma(N_b * \mu/c) * \text{Exp}[-N_b * \mu * (s/c) * \tau - \text{Exp}(-s * \tau)]$$

After establishment, clones grow exponentially (cell number $n = c/s * \text{Exp}[s * (t - \tau)]$ with a given establishment time τ at a sampled time t). Thus, the frequency of the adaptive event at a sampled time (t) can be estimated using the density distribution of establishment time.

$$f = \int_0^t d\tau * s/\Gamma(N_b * \mu/c) * \text{Exp}[-N_b * \mu * (s/c) * \tau - \text{Exp}(-s * \tau)] * c/(N_b * s) * \text{Exp}[s * (t - \tau)]$$

Lastly, by isolating I clones from the evolutionary experiment, we can estimate the probability of sampling this adaptive event.

$$\text{Prob}(\text{sampling this adaptive event}) = 1 - B(x = 0, \text{size} = I, p = f)$$

$B(x=0, \text{size}=I, p=f)$ represents a binomial sampling process -- given the probability of sampling the adaptive event (f) and the number of clones sampled (I), the probability of missing this adaptive clone during sampling. We use this binomial sampling because the number of adaptive clones sampled is not important in the calculation. Instead, whether or not the adaptive clone is sampled is more important.

Combining these three steps together, we can calculate the probability to sample this particular adaptive event in one equation:

$$\text{Prob}(\text{sampling the adaptive event}) = \int_0^t d\tau * s/\Gamma(N_b * \mu/c) * \text{Exp}[-N_b * \mu * (s/c) * \tau - \text{Exp}(-s * \tau)] * (1 - B(x = 0, \text{size} = I, p = c/(N_b * s) * \text{Exp}[s * (t - \tau)]))$$

11.2 Parameters—In our experiment, during each serial transfer cells are bottlenecked and then grown up for $T \approx 8$ generations. As described in the Supplementary Information in Levy, Blundell *et al*, the probability of the adaptive mutation arising during the first division of the cycle and surviving the bottleneck is $\approx N_b * u$. It is twice as likely to occur during the second division, but half as likely to survive the bottleneck, so the probability of a mutation entering at some point in the cycle and being present at a certain cell number once it is transferred to the next cycle is largely independent of where in the cycle it arises, always being proportional to $N_b * u$ with $N_b = 7E^{20}$.

The variance ($2c$) in offspring number through the cycle (including Poisson noise of growth and bottleneck) was denoted as ~ 3.5 (Supplementary Information in Levy, Blundell *et al*²⁰).

Assuming Poisson noise each generation, the effective variance in offspring number *per generation* would be $2c = 3.5/T^{20}$.

Furthermore, ~90 neutral clones were isolated from Evo2D at generation 88 and sequenced in Venkataram, Dunn *et al*²¹. Based on the number of mutations in their genomes, we estimated a mutation rate (μ) ~9E-10 per base-pair (bp) per division.

11.3 Probability of not observing an optimal type with a 1bp mutational target size—Assume that there is *1bp* in the yeast genome which can be mutated to the optimal type. We want to estimate the probability of sampling such an adaptive event from our evolutionary conditions. We consider the conservative scenario where the optimal type can only be generated by a specific nucleic acid change. Thus, the mutation rate $\mu = 9E-10/3 = 3E-10$ will be used for the following calculation.

Take the optimal type in Evo2D as an example. Based on its performance in fermentation and respiration, we can estimate its fitness per growth cycle in Evo2D ($s = 16h * \text{fermentation performance} + 28h * \text{respiration performance} = 2.56 \text{ per growth cycle}$). As cells grow up to 8 generations per cycle, therefore, its *fitness per generation* is $s/8$. Two replicates were conducted for Evo2D with 3,840 clones being isolated from replicate 1 and 960 clones being isolated from replicate 2. Using above parameters and equations, we can estimate that if such a 1bp mutational target size exists, the probabilities to sample this mutant from Evo2D replicate 1 and replicate 2 are 0.82 and 0.80, respectively.

Similarly, the probabilities to observe an *1bp* optimal mutational event in Evo1D ($s = 16h * \text{fermentation performance} + 4h * \text{respiration performance} = 1.49 \text{ per growth cycle}$) and Evo5D ($s = 40h * \text{respiration performance} + 60 * \text{stationary phase performance} = 2.98 \text{ per growth cycle}$) are estimated. Note that the fitness of Evo5D optimal type is estimated by assuming a fermentation performance zero. As most adaptive clones do gain benefits from performance, the fitness of Evo5D optimal type is very likely to be under-estimated, providing a conservative calculation in our case. Two replicates were conducted for each evolutionary condition with 800 clones being isolated from each replicate. With a *1bp* mutational target size, the probability to sample this mutant from either Evo1D replicate 1 or replicate 2 is 0.39, the probability to sample this mutant from either Evo5D replicate 1 or replicate 2 is 0.87.

Based on above calculation, if the ancestral yeast is able to be mutated to maximize fermentation and respiration performances simultaneously by a single-step mutation, the probability of not observing such a mutant in both replicates of Evo1D and Evo2D will be $p = (1-0.39)*(1-0.39)*(1-0.82)*(1-0.8) = 0.013$.

Similarly, if the ancestral yeast is able to be mutated to maximize respiration and stationary phase performances by a single-step mutation, the probability of not observing such a mutant in both replicates of Evo5D will be $p = (1-0.87)*(1-0.87) = 0.017$.

Due to the difficulty in measuring fitness in Evo1/5D, the probability to not sample such optimal types is not calculated. If Evo1/5D was considered, the chance to miss such optimal types would be even smaller than what we estimated above.

11.4 A conservative estimate of the above probability—In our previous study (Li, Venkataram *et al*²²), we noticed an inflation of fitness in one batch of fitness measurements; nonetheless, fitness measurements across batches are high correlated (Supplementary Fig. 5). This batch of fitness measurements is used to infer fermentation and respiration performances among Evo2D adaptive clones in this study. Thus, the fitness estimate of the optimal type in the fermentation and respiration space may be affected and also inflated. Fitness measurements of Evo1D, Evo5D, and Evo1/5D adaptive clones were conducted independently in a different batch. Thus, these measurements likely don't suffer such an inflation.

However, to have a conservative estimate, we corrected the estimated fitness of all optimal types by an inflation factor ($s_{correct} = (s + 0.01)/1.472$, Supplementary Fig. 5) and generated corrected fitness estimates: *s per cycle* of Evo2D optimal type = 1.75, *s per cycle* of Evo1D optimal type = 1.02, and *s per cycle* of Evo5D optimal type = 2.04.

With corrected fitness estimates, the probabilities to sample a single-mutation optimal type in Evo2D replicate 1 and replicate 2 are 0.6 and 0.54, respectively. The probability to sample a single-mutation optimal type in either replicate of Evo1D is 0.08. The probability to sample a single-mutation optimal type in either replicate of Evo5D is 0.65.

Thus, if the ancestral yeast strain is able to be mutated to maximize fermentation and respiration performances simultaneously by a single-step mutation, the probability of not observing such a mutant in both replicates of Evo1D and Evo2D will be $p = (1-0.08)*(1-0.08)*(1-0.6)*(1-0.54) = 0.156$.

If the ancestral yeast is able to be mutated to maximize respiration and stationary phase performances by a single-step mutation, the probability of not observing such a mutant in both replicates of Evo5D will be $p = (1-0.65)*(1-0.65) = 0.123$, still strongly suggesting a lack of such a single-step mutation in the ancestral yeast genome.

12 Viability Measurements

Two mutants carrying *FPK1* mutations, with one of them carrying an additional chromosome IX duplication, and two independent WT strains were cultured in monoculture for 5 days for viability measurements. The number of viable cells which formed colonies on plates was divided by the expected number measured by Coulter Counter to calculate viability -- the fraction of clones that are viable. Viability tests were conducted in two independent replicates with consistent results (Supplementary Table 4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We wish to thank A. Agarwala, G. Kinsler, C. McFarland, and D. Fisher for discussion. We thank J. Blundell, K. Geiler-Samerotte, O. Kolodny, S. Kryazhimskiy, C. Li, F. Rosenzweig and S. Venkataram for comments on the manuscript. We thank all members in the Sherlock and Petrov labs for helpful suggestions. Y.L. is supported by the Stanford Center for Computational, Human and Evolutionary Genomics (CEHG) Predoctoral Fellowship. The work

was supported by NIH grants R01 GM110275 and R35 GM131824 and NASA grant NNX17AG79G to G.S. and NIH grant R35GM118165 to D.A.P..

References

1. Loschiavo SR EFFECT OF OVIPOSITION SITES ON EGG PRODUCTION AND LONGEVITY OF TROGODERMA PARABILE (COLEOPTERA: DERMESTIDAE)1. *Can. Entomol.* 100, 86–89 (1968).
2. Tinkle DW The Concept of Reproductive Effort and Its Relation to the Evolution of Life Histories of Lizards. *Am. Nat.* 103, 501–516 (1969).
3. Reznick DA, Bryga H & Endler JA Experimentally induced life-history evolution in a natural population. *Nature* 346, 357–359 (1990).
4. Stearns SC The Evolution of Life Histories. (Oxford University Press, 1992).
5. Camargo A, Sarroca M & Maneyro R Reproductive effort and the egg number vs. size trade-off in *Physalaemus* frogs (Anura: Leiuperidae). *Acta Oecologica* 34, 163–171 (2008).
6. Cunningham JT Degenerative Mutations. *Nature* 130, 203–204 (1932).
7. Wang Y et al. Contribution of both positive selection and relaxation of selective constraints to degeneration of flyability during geese domestication. *PLOS ONE* 12, e0185328 (2017).
8. Darwin C The Descent of Man, and Selection in Relation to Sex. *Princeton University Press* (1871). Available at: <https://press.princeton.edu/titles/414.html>. (Accessed: 12th January 2019)
9. Shoval O et al. Evolutionary Trade-Offs, Pareto Optimality, and the Geometry of Phenotype Space. *Science* 336, 1157–1160 (2012). [PubMed: 22539553]
10. Tendler A, Mayo A & Alon U Evolutionary tradeoffs, Pareto optimality and the morphology of ammonite shells. *BMC Syst. Biol.* 9, 12 (2015). [PubMed: 25884468]
11. Mooney KA, Halitschke R, Kessler A & Agrawal AA Evolutionary Trade-Offs in Plants Mediate the Strength of Trophic Cascades. *Science* 327, 1642–1644 (2010). [PubMed: 20339073]
12. Fraebel DT et al. Environment determines evolutionary trajectory in a constrained phenotypic space. *eLife* 6, e24669 (2017).
13. Nidelet T & Kaltz O Direct and correlated responses to selection in a host-parasite system: testing for the emergence of genotype specificity. *Evol. Int. J. Org. Evol.* 61, 1803–1811 (2007).
14. Buckling A, Brockhurst MA, Travisano M & Rainey PB Experimental adaptation to high and low quality environments under different scales of temporal variation. *J. Evol. Biol.* 20, 296–300 (2007). [PubMed: 17210022]
15. Bono LM, Smith LB, Pfennig DW & Burch CL The emergence of performance trade-offs during local adaptation: insights from experimental evolution. *Mol. Ecol.* 26, 1720–1733 (2017). [PubMed: 28029196]
16. McGee LW et al. Synergistic Pleiotropy Overrides the Costs of Complexity in Viral Adaptation. *Genetics* 202, 285–295 (2016). [PubMed: 26564159]
17. Jasmin J-N & Kassen R On the experimental evolution of specialization and diversity in heterogeneous environments. *Ecol. Lett.* 10, 272–281 (2007). [PubMed: 17355566]
18. Bennett AF & Lenski RE An experimental test of evolutionary trade-offs during temperature adaptation. *Proc. Natl. Acad. Sci.* 104, 8649–8654 (2007). [PubMed: 17494741]
19. Satterwhite RS & Cooper TF Constraints on adaptation of *Escherichia coli* to mixed-resource environments increase over time. *Evolution* 69, 2067–2078 (2015). [PubMed: 26103008]
20. Levy SF et al. Quantitative evolutionary dynamics using high-resolution lineage tracking. *Nature* 519, 181–186 (2015). [PubMed: 25731169]
21. Venkataram S et al. Development of a Comprehensive Genotype-to-Fitness Map of Adaptation-Driving Mutations in Yeast. *Cell* 166, 1585–1596.e22 (2016). [PubMed: 27594428]
22. Li Y et al. Hidden Complexity of Yeast Adaptation under Simple Evolutionary Conditions. *Curr. Biol.* 28, 515–525.e6 (2018). [PubMed: 29429618]
23. Seedorf M & Silver PA Importin/karyopherin protein family members required for mRNA export from the nucleus. *Proc. Natl. Acad. Sci. U. S. A.* 94, 8590–8595 (1997). [PubMed: 9238021]

24. Rosenblum JS, Pemberton LF & Blobel G A Nuclear Import Pathway for a Protein Involved in tRNA Maturation. *J. Cell Biol.* 139, 1655–1661 (1997). [PubMed: 9412461]
25. Baccarini L, Martínez-Montañés F, Rossi S, Proft M & Portela P PKA-chromatin association at stress responsive target genes from *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta BBA - Gene Regul. Mech.* 1849, 1329–1339 (2015).
26. Yona AH et al. Chromosomal duplication is a transient evolutionary solution to stress. *Proc. Natl. Acad. Sci.* 109, 21010–21015 (2012). [PubMed: 23197825]
27. Natesuntorn W et al. Genome-wide construction of a series of designed segmental aneuploids in *Saccharomyces cerevisiae*. *Sci. Rep.* 5, 12510 (2015). [PubMed: 26224198]
28. Sunshine AB et al. The Fitness Consequences of Aneuploidy Are Driven by Condition-Dependent Gene Effects. *PLOS Biol.* 13, e1002155 (2015).
29. Garay E et al. High-Resolution Profiling of Stationary-Phase Survival Reveals Yeast Longevity Factors and Their Genetic Interactions. *PLOS Genet.* 10, e1004168 (2014).
30. Levins R Theory of Fitness in a Heterogeneous Environment. I. The Fitness Set and Adaptive Function. *Am. Nat.* 96, 361–373 (1962).
31. Ehrlich E, Kath NJ & Gaedke U The shape of a defense-growth trade-off governs seasonal trait dynamics in natural phytoplankton. *bioRxiv* 462622 (2018). doi:10.1101/2622
32. Jessup CM & Bohannan BJM The shape of an ecological trade-off varies with environment. *Ecol. Lett.* 11, 947–959 (2008). [PubMed: 18557986]
33. Maharjan R et al. The form of a trade-off determines the response to competition. *Ecol. Lett.* 16, 1267–1276 (2013). [PubMed: 23902419]
34. Roff DA & Fairbairn DJ The evolution of trade-offs: where are we? *J. Evol. Biol.* 20, 433–447 (2007). [PubMed: 17305809]
35. Yi X & Dean AM Phenotypic plasticity as an adaptation to a functional trade-off. *eLife* 5, e19307 (2016).
36. Sexton JP, Montiel J, Shay JE, Stephens MR & Slatyer RA Evolution of Ecological Niche Breadth. *Annu. Rev. Ecol. Evol. Syst.* 48, 183–206 (2017).
37. Zhao L, Liu Z, Levy SF & Wu S Bartender: a fast and accurate clustering algorithm to count barcode reads. *Bioinformatics* 34, 739–747 (2018). [PubMed: 29069318]
38. Kryazhimskiy S, Rice DP, Jerison ER & Desai MM Global epistasis makes adaptation predictable despite sequence-level stochasticity. *Science* 344, 1519–1522 (2014). [PubMed: 24970088]
39. Martin M Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* 17, 10–12 (2011).
40. Li H Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *ArXiv13033997 Q-Bio* (2013).
41. Freed DN, Aldana R, Weber JA & Edwards JS The Sentieon Genomics Tools - A fast and accurate solution to variant calling from next-generation sequence data. *bioRxiv* 115717 (2017). doi: 10.1101/115717
42. Cingolani P et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff. *Fly (Austin)* 6, 80–92 (2012). [PubMed: 22728672]

Figure 1: Evolutionary constraints in trait-performance space.

a, The Pareto optimality front separates the evolutionary accessible (white space) from the inaccessible space (shaded space). The red dot represents mutants that maximize both traits simultaneously. When organisms are on the Pareto optimality front (green dots), increasing the performance for one trait decreases the performance for the other. By contrast, when organisms are behind the Pareto front (black dot), organisms can improve the performance of both traits until the front is reached. **b,** An organism on a three-dimensional Pareto surface (green dot) appears to be sub-optimal when it is projected onto a two-dimensional space. **c-d,** When the ancestor (Anc) is behind the Pareto front, many individuals occupying different parts of the trait space (**c**) are required to characterize the Pareto front. By contrast, too few individuals (**d**) are insufficient to delineate the front.

Figure 2: Experimental design and the observation of local adaptation and tradeoffs.

a, Three chosen evolutionary conditions span different phases of the yeast growth cycle. Clones were also evolved in a 1-day/5-day alternating condition (Evo1/5D). **b**, Fitness measurement conditions designed to quantify fermentation, respiration and stationary performances (fitness change per hour) of each clone. Dashed vertical lines separate different growth phases, colored as **(a)**. **c**, Fitness measurements of adaptive clones, grouped by their “home” evolutionary condition, in “home” and “away” conditions. Arrows point to adaptive clones measured in their “home” condition. The lower and upper hinges of each

box correspond to the first and third quartiles (the 25th and 75th percentiles). The whiskers extend from the hinge to a value no further than $1.5 * \text{IQR}$ from the hinge (where IQR is the inter-quartile range). The width of the violin represents the probability density of the data at different fitness values. **d**, Adaptive clones' fermentation, respiration and stationary performances grouped by their evolutionary condition. +/- indicates increased/decreased performance compared to the ancestor. **e**, Clones are separated by their evolutionary condition and colored by their stationary phase performance. Each dot represents a clone. Note that some blue colored clones from Evo5D and Evo1/5D (pointed by arrows) improve performances in all three growth phases.

Figure 3: Mapping of the evolutionarily accessible trait space.

For each pair of performances (fitness change per hour in each growth phase), adaptive clones are plotted and colored by either their molecular basis (a-c), or their evolutionary conditions (d-f). Each dot represents a clone. The large red dots represent the optimum phenotypes, achieving the upper limits (dashed lines) of each pair of performances. The grey curves, defined by the convex hull algorithm, represent putative Pareto optimality fronts. d-f, Histograms on the side represent the density distribution of each trait's performance. Based on the null distribution, the p-value of observing an empty space between the putative front and the optimal type (the large red dot) as large as observed in the real data is reported.

Figure 4: Pareto front geometry, and possible changes over longer-term evolution.

a-b, (a) the convex-shaped Pareto front favors generalists, while (b) the concave-shaped front favors specialists during evolution. **c-f,** The current convex Pareto front (the solid grey curve) can (c) change into a rectangle, with the previously inaccessible space being populated, (d) stay in place, (e) move forward while keeping its shape, and (f) change its shape over longer-term evolution. Potential Pareto fronts after longer-term evolution are depicted in orange dashed lines.

Table 1:

Genetic basis of adaptation and tradeoffs The number of clones carrying recurrent mutations within genes or pathways. These genes/pathways were independently mutated more than four times. Genes in the same pathway are grouped by the large parenthesis on the left.

		Evo1D	Evo2D	Evo5D	Evo1/5D
RAS/PKA	CYR1	0	3	1	1
	GPB2	0	13	0	0
	RAS2	0	2	0	0
	IRA1	0	39	0	0
	IRA2	0	10	1	0
	PDE2	0	11	0	0
TOR/Sch9	TOR1	0	1	0	0
	SCH9	0	1	0	0
	SXM1	12	1	10	26
	CHR11 DUP	0	4 (diploid)	11	1
HOG	FPK1	0	1	10	2
	SSK1	0	0	7	0
	SSK2	0	2	1	0
	HOG1	0	0	1	0