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Publication Date

2022

DOI

10.3389/fevo.2022.859904

Peer reviewed

Temperature-dependent genetics of thermotolerance between yeast species

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Abstract

Many traits of industrial and basic biological interest arose long ago, and manifest now as fixed differences between a focal species and its reproductively isolated relatives. In these systems, extant individuals can hold clues to the mechanisms by which phenotypes evolved in their ancestors. We harnessed yeast thermotolerance as a test case for such molecular-genetic inferences. In viability experiments, we showed that extant *Saccharomyces* cerevisiae survived at temperatures where cultures of its sister species *S. paradoxus* died out. Then, focusing on loci that contribute to this difference, we found that the genetic mechanisms of high-temperature growth changed with temperature. We also uncovered an enrichment of low-frequency variants at thermotolerance loci in *S. cerevisiae* population sequences, suggestive of a history of non-neutral selective forces acting at these genes. We interpret these results in light of models of the evolutionary mechanisms by which the thermotolerance trait arose in the *S. cerevisiae* lineage. Together, our results and interpretation underscore the power of genetic approaches to explore how an ancient trait came to be.

Keywords

Saccharomyces ; Ancient; Evolution; Thermotolerance; Cline; Adaptation

1 Introduction

A central goal of evolutionary genetics is to understand how nature builds new phenotypes. Thanks to advances in statistical genetics and experimental evolution, mechanisms of trait evolution over relatively short timescales have come well within reach in the modern literature. By contrast, longer-term innovations have posed a more profound challenge for the field (Orr, 2001). In principle, for a phenotype that originated long ago and manifests now as a fixed difference between species, evolution could have refined the character along the entire divergence time of the respective taxa. In landmark cases, candidate-gene studies

have shed light on suites of mutational changes of this kind between species, at a given model locus. This includes the order by which adaptive alleles were likely acquired, and/or the functional pressures that drove them (Bridgham et al., 2009; Baldwin et al., 2014; Dong et al., 2014; Sayou et al., 2014; Anderson et al., 2015; Daugherty et al., 2016; Sulak et al., 2016; Liu et al., 2018; Starr et al., 2018; Xie et al., 2018; Siddiq and Thornton, 2019; Pillai et al., 2020; Prieto-Godino et al., 2021). But, in most cases, any factor pursued by such a candidate-gene approach only represents part of the complex genetic architecture of an ancient trait. As a field, we still know relatively little about how evolution coordinates multiple adaptive loci over deep divergences.

In the search for evolutionary principles, genetically tractable model systems can be of great utility. *Saccharomyces* yeasts are well suited for this purpose, and environmental yeast isolates have been studied extensively for their innovations within and between species (Hittinger, 2013). Thermotolerance is of particular interest because it tracks with phylogeny across the 20 million years of the *Saccharomyces* radiation (Gonçalves et al., 2011; Salvadó et al., 2011). Even the two most recent branches of the phylogeny exhibit a robust difference in this phenotype: *S. cerevisiae* acquired the ability to grow at temperatures near 40°C in the five million years since it diverged from its sister species, *S. paradoxus* (Sweeney et al., 2004; Gonçalves et al., 2011; Salvadó et al., 2011; Williams et al., 2015). In previous work, a whole-genome mapping scheme identified housekeeping genes at which variation between *S. cerevisiae* and *S. paradoxus* impacts growth at the high end of the *S. cerevisiae* temperature range (Weiss et al., 2018). These loci exhibit striking sequence differences between the species, and conservation in *S. cerevisiae*, consistent with a history of positive selection on pro-thermotolerance alleles (Weiss et al., 2018; Abrams et al., 2021, 2022). However, we have as yet little insight into the ecological dynamics by which this model trait evolved along the *S. cerevisiae* lineage.

Cases of adaptation across a temperature gradient are a mainstay of the evolutionary genetics literature (Turner et al., 2008; Prasad et al., 2011; Mimura et al., 2013; Savolainen et al., 2013; Robin et al., 2017; Dudaniec et al., 2018; Key et al., 2018; Endler, 2020; Tepolt and Palumbi, 2020; Calfee et al., 2021; Machado et al., 2021). Here we sought to explore the relevance of such a mechanism to the events by which *S. cerevisiae* gained its maximal thermotolerance phenotype. Given that we have no access to genotypes representing ancient intermediates between this species and *S. paradoxus*, we designed a strategy to interrogate the genetics of extant strains, focusing on contributing genes of major effect. We surveyed gene-environment interactions by these thermotolerance loci across warm temperatures, complementing previous studies of interspecies variation at a single high temperature (Weiss et al., 2018; Abrams et al., 2022). And we investigated the frequency of variants at these loci with a population-genomic approach.

2 Methods

2.1 Viability assay

For the survey of viability phenotypes at high temperatures across wild-type isolates in Figure S1, strains were streaked out and a colony of each was pre-cultured in liquid as for 39°C growth above, except that the initial pre-culture to achieve saturation lasted 48

hours. Each pre-culture was back-diluted into 10 mL of YPD to reach an OD_{600}/mL of 0.05 and then cultured for 24 hours at the temperature of interest (28°C - 39°C). The viability of both the precultures and the cultures after 24 hours at the temperature of interest were measured with a spotting assay, where we diluted aliquots from the culture in a 1:10 series and spotting 3 μL of each dilution for growth on a solid YPD plate. After incubation at 28°C for two days, we used the dilution corresponding to the densest spot that was not a lawn for to determine viability: we counted the number of colonies in each of the two technical replicate spots, formulated the number of colony forming units per mL of undiluted culture (CFU/mL). We determined the change in viable cells by subtracting the number of cells in the culture at the initial time point from that at the final timepoint, based on the CFU/mL count and the culture volume. We evaluated the significance of the difference between *S. cerevisiae* and *S. paradoxus* at a given temperature using a one-sided Mann-Whitney U test.

2.2   Dose response growth assay

For growth measurements in Figure 1 we assayed *S. paradoxus* Z1; *S. cerevisiae* DBVPG1373; and *S. cerevisiae* DBVPG1373 with the *S. paradoxus* Z1 allele of *ESP1*, *MYO1*, *AFG2*, or *CEP3* swapped in at the endogenous locus from (Weiss et al., 2018) (Table S1) as follows. Each strain was streaked from a −80°C freezer stock onto a yeast peptone dextrose (YPD) agar plate and incubated at room temperature for 3 days. For each biological replicate, a single colony was inoculated into 5 mL liquid YPD and grown for 24 hours at 28°C with shaking at 200 rpm to generate pre-cultures. Each pre-culture was back-diluted into YPD at an OD_{600}/mL of 0.05 and grown for an additional 5.5–6 hours at 28°C, shaking at 200 rpm, until reaching logarithmic phase. Each pre-culture was again back-diluted into 10 mL YPD in 1-inch diameter glass tubes with a target OD_{600}/mL of 0.05; the actual OD_{600}/mL of each was measured, after which it was grown at a temperature of interest (28°C or 35–38°C) with shaking at 200rpm for 24 hours, and OD_{600}/mL was measured again. The growth efficiency for each replicate was calculated as the difference between these final and initial OD_{600}/mL values. We used the growth efficiency from all days and all temperatures of a given pair of strains as input into a two-factor type 2 ANOVA test for a temperature-by-strain effect (Table S2).

For growth measurements of *ESP1* swap strains with different donors, as reported in Figure S2, cultures were grown and measured as above, except that the only temperature was 36°C.

2.3   Tajima's D in Wine/European *S. cerevisiae*

Tajima's D tabulates the difference between the average number of differences in pairs of sequences in a population sample and the number of variant sites in the sample. When the latter is of much bigger magnitude and D is negative, it indicates that variation in the sample is accounted for mostly by rare alleles. This pattern is expected some time after a selective sweep when *de novo* mutations arise on the swept haplotype; it can also reflect weak purifying selection or a history of population expansion (Suzuki, 2010). Taking as input the VCFs reporting inheritance in the strains of a given *S. cerevisiae* population from (Peter et al., 2018), we used VCF-kit (Cook and Andersen, 2017) to calculate Tajima's D for the region from coding start to coding stop for each gene. We then developed a resampling test to assess enrichment trends in Tajima's D in genes of interest against a genomic null.

This scheme normalizes out impacts on Tajima's D values from events which affect the whole genome, such as a population expansion; that said, formally, any results from such an empirical outlier-based analysis serve as suggestive rather than conclusive evidence for non-neutral evolution (Teshima et al., 2006; Thornton and Jensen, 2007). For the test, we sampled 10,000 random cohorts of genes from the genome with the same number of essential and nonessential genes (Winzeler et al., 1999) as a given thermotolerance cohort, and we used as an empirical *P*-value the proportion of random cohorts where the median Tajima's D was less than or equal to that of our thermotolerance cohort.

3   Results

For an initial study of the genetics of yeast species variation in temperature response, we chose to harness DBVPG1373, a homozygous diploid *S. cerevisiae* strain derived from an isolate from Dutch soil, and Z1, a homozygous diploid *S. paradoxus* strain derived from an isolate from an oak tree in England. We anticipated that detailed analyses using these strains, as representatives of their respective species, could accelerate the discovery of more general principles (Weiss et al., 2018; Abrams et al., 2021, 2022). We developed an assay quantifying cell viability in a given liquid culture before and after incubation at a temperature of interest, and we implemented this approach for each species in turn. The results revealed an advantage for *S. cerevisiae* over *S. paradoxus* at temperatures above 35°C (Figure S1), consistent with previous growth-based surveys (Sweeney et al., 2004; Gonçalves et al., 2011; Salvadó et al., 2011; Williams et al., 2015). *S. cerevisiae* maintained viability at all temperatures tested, whereas by 37°C, *S. paradoxus* became inviable, with no evidence for spontaneous rescue even over long incubation times (Figure S1). Using the latter as a window onto the phenotype of the common ancestor of *S. cerevisiae* and *S. paradoxus*, we would envision that the latter ancient population would have gone extinct rather than adapting, if exposed to temperatures at the high end of the range tolerated by modern *S. cerevisiae*.

We next aimed to investigate the genetics of temperature response as it differs between extant *S. cerevisiae* and *S. paradoxus*, to motivate inferences about the evolution of the maximal thermotolerance trait. We focused on four genes—the cell division factors *ESP1*, *MYO1*, and *CEP3*, and the ribosome maturation factor *AFG2*—where alleles from modern *S. paradoxus* compromise growth at 39°C (Weiss et al., 2018). We made use of homozygous diploid strains of the *S. cerevisiae* DBVPG1373 background in which the endogenous allele of each gene in turn was replaced with that of *S. paradoxus* Z1. In each, we measured the growth phenotype as a dose-response across temperatures, and observed a drop as temperature increased (Figure 1). In the *S. cerevisiae* background, *S. paradoxus* Z1 alleles eroded growth efficiency at temperatures well below the hard limit of viability for the Z1 wild-type (~38°C), an effect that reached significance for three of our four focal genes (Figure 1). We conclude that many problems posed by *S. paradoxus* Z1 alleles at the high end of our temperature range also are relevant to a lesser extent at lower temperatures.

Inspecting the shape of the temperature dose-responses of allelic effects, we noted quantitative differences between our loci (Figure 1 and Table S2). At the chromatid separase gene *ESP1*, the Z1 allele conferred an appreciable drop in growth efficiency at 36°C and

supported almost no growth by 37°C. By contrast, at *AFG2*, the *S. paradoxus* Z1 allele was sufficient for growth approximating that of *S. cerevisiae* until 38°C. The dose-response of allelic effects at *MYO1*, encoding a class II myosin heavy chain, lay between these two extremes. Overall, all allele-replacement strains exhibited distinct dose-response behaviors across temperatures (Table S2), likely reflecting distinguishing properties of their structure and function, and of the interspecies variants they harbor.

We reasoned that trends from our temperature dose-response approach would be most informative when they were conserved across *S. paradoxus* as a species. To pursue this, we earmarked *ESP1*, whose *S. paradoxus* Z1 allele had exhibited the sharpest falloff with temperature among the genes of our set (Figure 1). We repeated our growth efficiency experiments in strains of *S. cerevisiae* DBVPG1373 harboring *ESP1* from other *S. paradoxus* donors beside the Z1 strain. These transgenics, which phenocopy wild-type DBVPG1373 at 28°C (Weiss et al., 2018), all dropped off in growth efficiency by 36°C (Figure S2), as expected if the temperature preference of *ESP1* were ancestral to, and shared across, extant *S. paradoxus* populations.

Together, these dose-response results establish that the functions of *S. paradoxus* alleles at our focal genes break down at distinct temperatures between 35°C and 38°C—suggesting similar gene-by-environment effects in the ancestor of *S. cerevisiae* and *S. paradoxus*, if it sampled a range of temperature conditions over evolutionary history.

To gain insight into past and current selective pressures on thermotolerance loci, we turned to a molecular-evolution approach. Previous work on *Saccharomyces* thermotolerance genes has emphasized sequence divergence between species (Weiss et al., 2018; Abrams et al., 2021, 2022). For a complementary focus on population variation within *S. cerevisiae*, we analyzed the Tajima's D statistic, which formulates properties of sequence variants in a population into a test of how well the sequence fits the expectations under a neutral evolutionary model (Tajima, 1989; Biswas and Akey, 2006). We first developed a resampling-based scheme that compared Tajima's D between a gene cohort of interest and a genomic null. This enabled a non-parametric assessment of significance and normalized out potential effects of demography on the statistic (see Methods). Expecting that our test would have maximal power in a data set of large sample size, we focused on the most deeply-sampled *S. cerevisiae* population in current compendia, comprising isolates from vineyards and European soil (362 strains; Peter et al., 2018). Examining our four focal thermotolerance genes, we detected an enrichment for low, negative Tajima's D at these loci across *S. cerevisiae* genomes ($P = 0.0263$; Figure S3 and Table S3A), reporting an excess of rare variants—as expected after a selective sweep, or under constraints from purifying selection (Biswas and Akey, 2006; Suzuki, 2010). We repeated this analysis using more comprehensive sets of hits from interspecies thermotolerance screens (Weiss et al., 2018; Abrams et al., 2021, 2022), and detected strong signal for low, negative Tajima's D at these loci in vineyard/European *S. cerevisiae* in every case (Table S3B–C). Interestingly, *ESP1* exhibited the most negative Tajima's D value among all thermotolerance genes in these analyses (Table S3), dovetailing with the strong effect of variation at this gene in phenotypic analyses (Weiss et al. 2018 and Figure 1).

We also investigated patterns of Tajima's D in thermotolerance genes beyond vineyard/ European *S. cerevisiae*. For this purpose we harnessed the next best-sampled *S. cerevisiae* populations from (Peter et al., 2018): Mosaic Region 3 (113 strains), Mixed Origin (72 strains), Sake (47 strains), and Brazilian Bioethanol (35 strains). Of these groups of isolates, tests for enrichment of low Tajima's D among our four focal thermotolerance genes yielded significant signal only in the Brazilian Bioethanol population (Table S3A). Thus, the trend for low, negative Tajima's D at thermotolerance loci was detectable but not consistent across these populations, potentially reflecting limited power, or weaker or different selective forces, in the latter relative to vineyard/European strains.

4 Discussion

Most trait differences that define long-diverged species are likely the product of suites of unlinked variants that have come together over long timescales. Tracing the ancient evolutionary events at such loci remains a central challenge in the field. In this work, we have characterized thermotolerance genes in extant yeast strains and species, to inform models of the evolution of the trait in *S. cerevisiae*.

Our data have shown that, for the strains studied here, temperatures below the high end of the *S. cerevisiae* range are lethal for *S. paradoxus*. This complements previous surveys of the *Saccharomyces* clade using growth-based assays (Sweeney et al., 2004; Gonçalves et al., 2011; Salvadó et al., 2011) and reveals that, under high temperature conditions, a given culture of *S. paradoxus* will die off rather than adapting. We have also leveraged results from genetic mapping of *S. cerevisiae* thermotolerance to trace the temperature dependence of contributing genes. Our approach contrasts with the search for DNA sequence variants associated with environment or population variables (Rellstab et al., 2015; Hoban et al., 2016), in that we focus directly on bona fide causal determinants of the trait of interest. With this strategy, we discerned differences between thermotolerance loci in terms of the temperatures at which alleles from modern *S. paradoxus* fail in their growth functions. Our experiments for this purpose used transgenesis of one locus at a time in a single strain background, so the potential for epistasis involving *S. paradoxus* alleles remains untested. As they stand, our data do make clear that the genetic mechanisms of growth change with temperature in the *S. cerevisiae-S. paradoxus* system.

If we infer that these effects are representative of the genetics of a thermosensitive *S. cerevisiae* ancestor, long ago in history, we can formulate models of the mechanism by which thermotolerance evolved. In one scenario, an ancestral population of *S. cerevisiae* in temperate conditions could have acquired pro-thermotolerance alleles at many loci, which, once they had accumulated as standing variation, would have enabled a single jump to a hotter growth regime. Interestingly, however, experimental evolution studies suggest that events of the latter kind may be rare; by contrast, adaptation to a gradually increasing stress is evolutionarily easier and reduces the risk of extinction (Lindsey et al., 2013). Under a model of gradual adaptation, we would picture the ancestral *S. cerevisiae* population expanding its range stepwise along a temperature gradient, analogous to previous local-adaptation case studies in other systems (Mimura et al., 2013; Robin et al., 2017; Dudaniec et al., 2018; Key et al., 2018; Tepolt and Palumbi, 2020). As evolution proceeded along the

S. cerevisiae lineage, *ESP1*, *MYO1*, and other underlying loci would each have come under selective pressure (and acquired variants *de novo*) at the temperatures where their defects manifested. This would represent an example of "whack-a-mole" dynamics (Shin and MacCarthy, 2015), whereby the weakest point in the genetic network targeted by evolution changes as conditions change. Given the genetic complexity of the thermotolerance trait (Weiss et al., 2018), its history may ultimately prove to involve both gradual adaptation and sudden jumps to new resistance states, each relevant for some subset of the underlying alleles.

What were the ecological forces driving the gain of thermotolerance in *S. cerevisiae*? The ancestral population could have undergone a change in the temperature of its growth conditions over space or time: that is, migrants from temperate physical locales could have advanced to warmer sites, or the environment could have warmed up around a static population. In either case, it is tempting to speculate that these ecological pressures were exerted in the hot East Asian niches to which the ancestor of modern *S. cerevisiae* has been traced (Peter et al., 2018). Features of human-associated environments could also have contributed later, in domesticated populations. Alternatively, the trait syndrome in *S. cerevisiae*—a unique ability to tolerate ethanol as well as heat, with both given off by fermentative metabolism—could have arisen as a specialization to kill off microbial competitors in nutrient-rich substrates, regardless of the endemic temperature of the location (Goddard, 2008; Salvadó et al., 2011). If so, variants would have been acquired, potentially over millions of generations, gradually to ratchet up fermentative activity and tolerance to its byproducts, with our genetic insights thus far largely restricted to the latter.

Our work leaves open the dating of any such events. In several *S. cerevisiae* populations, we have uncovered an enrichment of rare variants at thermotolerance genes. This signature of non-neutral evolution can be interpreted at a given locus as evidence for a relatively recent sweep of a positively selected haplotype, or for negative selection to maintain a fitness-relevant haplotype that arose long ago. We favor the latter hypothesis, given that prior work across populations has also shown *S. cerevisiae* alleles at our focal genes to be partly sufficient for maximal thermotolerance, conserved within the species, and divergent from *S. paradoxus* (Weiss et al., 2018; Abrams et al., 2022). We thus propose that many thermotolerance alleles were acquired in ancient selective sweeps, before the radiation of modern *S. cerevisiae*, and have been maintained since then by purifying selection. That said, later refinements in particular populations of *S. cerevisiae* may also have strengthened beneficial facets of the trait, added regulatory tuning, or eliminated antagonistic pleiotropic "side effects" that were niche-specific. A comprehensive genetic and ecological reconstruction of this history may be out of our current grasp, especially in light of the caveats of our approach using a laboratory setting and extant strain backgrounds. Nonetheless, our data add compelling detail to an emerging consensus view of how evolution built maximal thermotolerance in *S. cerevisiae*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Data Availability Statement

Strains and plasmids are available upon request. Custom scripts for Tajima's D analyses are available at https://github.com/melanieabrams-pub/.

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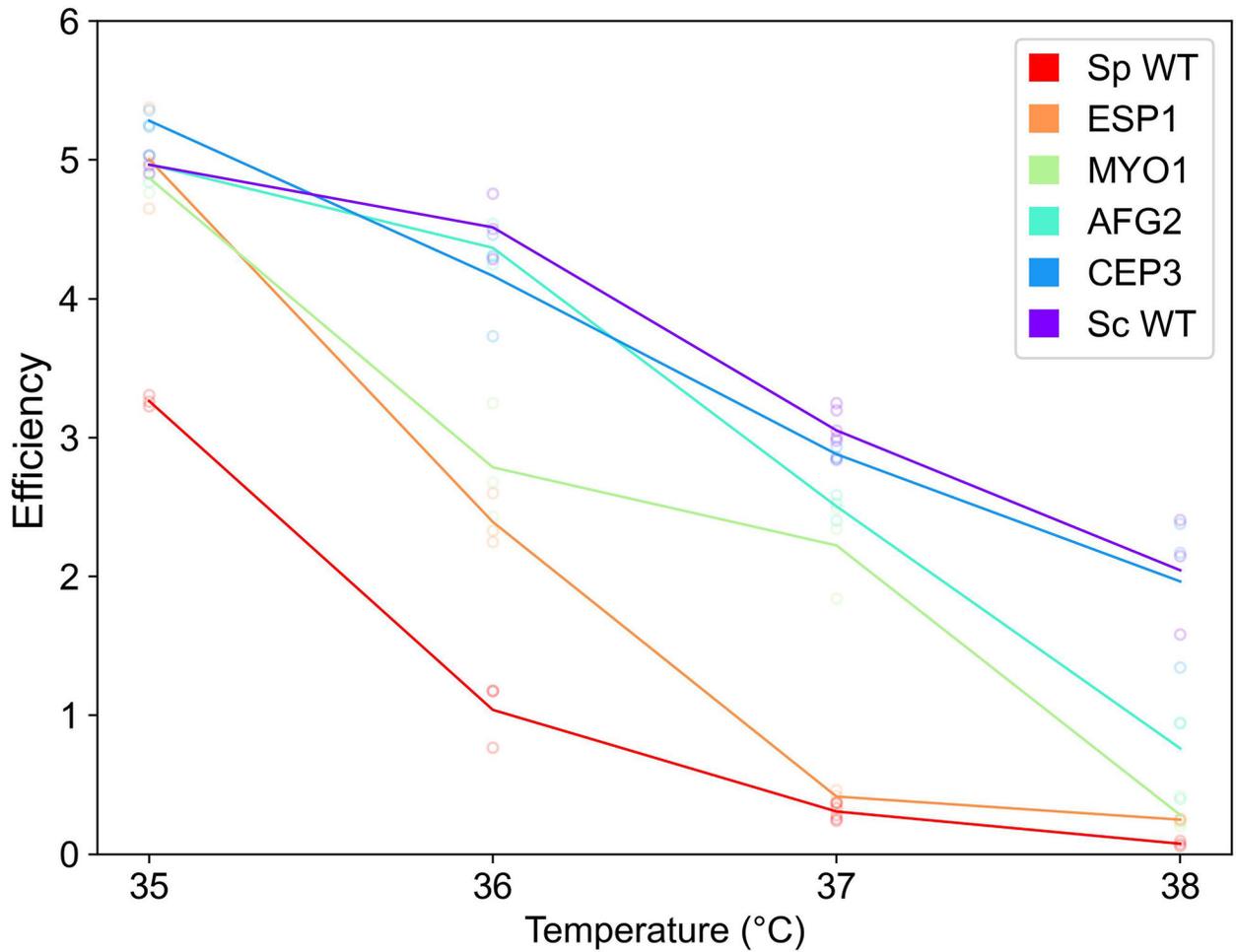
Figure 1. *S. paradoxus* alleles of thermotolerance genes confer temperature-dependent defects in the *S. cerevisiae* background.

Each line reports the results of growth experiments of a strain of *S. cerevisiae* DBVPG1373 harboring the indicated gene from *S. paradoxus* Z1, or the respective wild-type parent strains (WT), across a temperature gradient. The *y*-axis reports growth efficiency, the optical density reached by the culture after 24 hours at the temperature indicated on the *x*-axis, as a difference from the analogous quantity at time zero. Each point reports results from one biological replicate ($n = 3$), and the line represents the average growth efficiency of the indicated strain across the temperature gradient. *, $P < 10^{-3}$ for the strain by temperature interaction term of a two-factor ANOVA, in a comparison between the indicated strain and wild-type *S. cerevisiae* (Table S2).