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Fitness of evolving bacterial populations is contingent on deep and shallow history but only shallow history creates predictable patterns

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Long-term evolution experiments have tested the importance of genetic and environmental factors in influencing evolutionary outcomes. Differences in phylogenetic history, recent adaptation to distinct environments and chance events, all influence the fitness of a population. However, the interplay of these factors on a population's evolutionary potential remains relatively unexplored. We tracked the outcome of 2000 generations of evolution of four natural isolates of *Escherichia coli* bacteria that were engineered to also create differences in shallow history by adding previously identified mutations selected in a separate long-term experiment. Replicate populations started from each progenitor evolved in four environments. We found that deep and shallow phylogenetic histories both contributed significantly to differences in evolved fitness, though by different amounts in different selection environments. With one exception, chance effects were not significant. Whereas the effect of deep history did not follow any detectable pattern, effects of shallow history followed a pattern of diminishing returns whereby fitter ancestors had smaller fitness increases. These results are consistent with adaptive evolution being contingent on the interaction of several evolutionary forces but demonstrate that the nature of these interactions is not fixed and may not be predictable even when the role of chance is small.

1. Introduction

Evolutionary outcomes are determined by core factors that shape the diversity of life: adaptation, chance and history [1]. Adaptation reflects the power of natural selection to drive populations along evolutionary paths to phenotypes of high fitness. If few paths are available, replicate populations will follow repeatable, perhaps even predictable, outcomes [2–5]. By contrast, chance and history promote evolutionary divergence. Chance causes divergence between populations through stochastic differences in the occurrence and success of newly arising mutations [6–9]. History, defined here as differences in the genetic starting points of selected populations, promotes divergence if evolutionary opportunities or constraints are contingent on specific genotypes [10–15]. Determining the relative contribution of these forces, and how this might depend on the selective environment, is crucial to the goal of predicting evolutionary outcomes.

Chance events are an unavoidable part of evolution. Nevertheless, the effect of chance may be overwhelmed if there are only a limited number of available beneficial changes, leaving adaptation as the dominant force in determining

evolutionary outcomes. In this view, though there may be distinct routes to an evolutionary outcome, different paths will tend to converge [16–18]. Examples include the independent evolution of functionally similar eyes [19], toxins [20] and electric organs [21]. Alternatively, genetic interactions—epistasis—that cause the selective benefit of mutations to depend on the genetic background on which they occur can amplify the effect of mutational differences [3,15,22–26]. These interactions can lead to repeatably different evolutionary outcomes depending on a starting genotype when they depend on mutations that are distinct between those genotypes. They can also lead to divergence between replicate populations started from the same genotype when they depend on new mutations arising during evolution. Together, the effects of chance and history make the evolutionary dynamic much more complex than a repeatable process of optimization.

The contributions of adaptation, chance and history to evolution have largely been investigated by examining populations that have evolved under similar selective pressures [1]. For example, independent populations of Anolis lizards have converged on similarly selected morphologies [27]. However, even as similar selection pressures lead to similar outcomes, populations with distinct genetic starting points have evolved distinct phenotypes [28]. The complication of initial differences between evolving populations can be controlled for in laboratory experiments that start with genetically identical replicate populations. Analysis of a long-term experiment evolving initially identical replicate populations of *Escherichia coli* found similar overall changes in fitness, though with significant and sustained variation [29,30]. Moreover, one population evolved a novel phenotype via an evolutionary path that depended on a series of earlier mutations [10,31,32]. Other studies have found divergence among replicated populations dependent on the selective environment [33–35].

Laboratory studies that have tracked the evolution of populations starting with distinct genotypes have typically found that this history had a significant influence on evolutionary outcomes [11,15,25,26,36–44]. Of note, a significant proportion of this effect often depends on the starting fitness of a population, following a pattern of diminishing returns whereby fitter populations adapt more slowly than less fit populations [25,26,41,45,46]. Several studies have also identified genotypes that have significant differences in their evolutionary potentials that are not determined by initial fitness [11,36,47]. Indeed, analyses of mutational pathways have sometimes discovered intermediate genotypes crucial to determining future evolutionary outcomes [31,48,49].

A common limitation of studies examining the role of history on evolutionary outcomes is a focus on closely related populations that differ only by mutations acquired during short periods of laboratory adaptation [25,26,43]. High relatedness of starting populations probably reduces the potential influence of history on evolution because newly arising mutations will be tested on similar genetic backgrounds. The more diverse genetic backgrounds of individuals isolated from natural populations increases the chance that they will explore different regions of a fitness landscape during evolution, leading to divergent outcomes [11]. Analyses of evolving populations founded from individuals sampled directly from natural populations have found significant effects of history, though this tends to be weaker for the traits most closely related to fitness [50]. To our knowledge, however, no study has compared the contribution of historical effects at different levels of divergence, as is needed to assess how rapidly history can redirect evolutionary outcomes.

In this study, we compared the roles of adaptation, chance and history on the evolution of populations of *E. coli*. Four natural isolate strains of *E. coli* (deep history) were each engineered with three beneficial mutations (shallow history). Each strain–mutation combination was evolved with threefold replication (chance) for 2000 generations in four resource environments (figure 1). This crossed and replicated design allowed us to partition the variation in evolutionary outcomes into the effects of deep and shallow history, adaptation and chance.

2. Material and methods

(a) Bacterial strains and media

In a previous study, 26 natural isolate strains of *E. coli* were isolated and engineered by adding between one and four mutations [52]. We chose four of these natural isolates as progenitor strains (E704, B921, B175 and FBGM17; representing 'deep history') that each had three mutations (representing 'shallow history') separately added to comprise a set of 16 founder strains (figure 1). Mutations were added in the following genes or gene regions: *spot*, *pykF* and *rbs*. These mutations occurred early in the evolution of an *E. coli* population selected in minimal medium supplemented with glucose and conferred a significant fitness benefit in the background in which they arose [24,51]. At least the *spoT* and *pykF* mutations are likely to be highly pleiotropic, impacting core regulatory (*spoT*) or metabolic (*pykF*) processes, and therefore are good candidates for interacting with newly arising mutations to influence available evolutionary outcomes [23,53].

Populations started with founder strains were propagated in Davis-Mingioli minimal medium supplemented with glucose (0.5 g l^{-1}), casamino acids (1.4 g l^{-1}), acetate (0.36 g l^{-1}) or trypsin (0.064 g l^{-1}). These four resource environments were chosen following previous work [46,51,54]. Glucose was chosen as a reference because it is a preferred resource for *E. coli* frequently used in other long-term evolution studies [46,51]. Casamino acids are a mixture of amino acids that provides a rich growth environment. Acetate is a weak acid that is a by-product of *E. coli* growth in glucose [55]. Trypsin is a protein that must be degraded prior to uptake and was expected to impose a stressful low-nutrient environment.

(b) Experimental design

We carried out a long-term evolution experiment to investigate the relative contribution of adaptation, deep and shallow history, chance and selection environment, on the evolution of fitness. Populations were founded with each of the 16 strains described above, giving a crossed design of shallow history (added mutation) by deep history (progenitor natural isolate strain). Each strain was used to start 12 replicate populations, with three of these evolved in each of the four different resource environments, giving a total of 192 evolving populations (figure 1). Each population was propagated at 37°C for 2000 generations in unshaken test tubes with daily transfer of 5 µl to 5 ml of fresh medium.

(c) Competitive fitness assays

We performed one-day competitive fitness assays to quantify the relative fitness of evolved strains. The 16 founder strains we used were able to grow on arabinose (Ara+) and otherwise isogenic Ara- derivatives were isolated previously [52]. These two sets of strains can be distinguished visually on tetrazolium arabinose

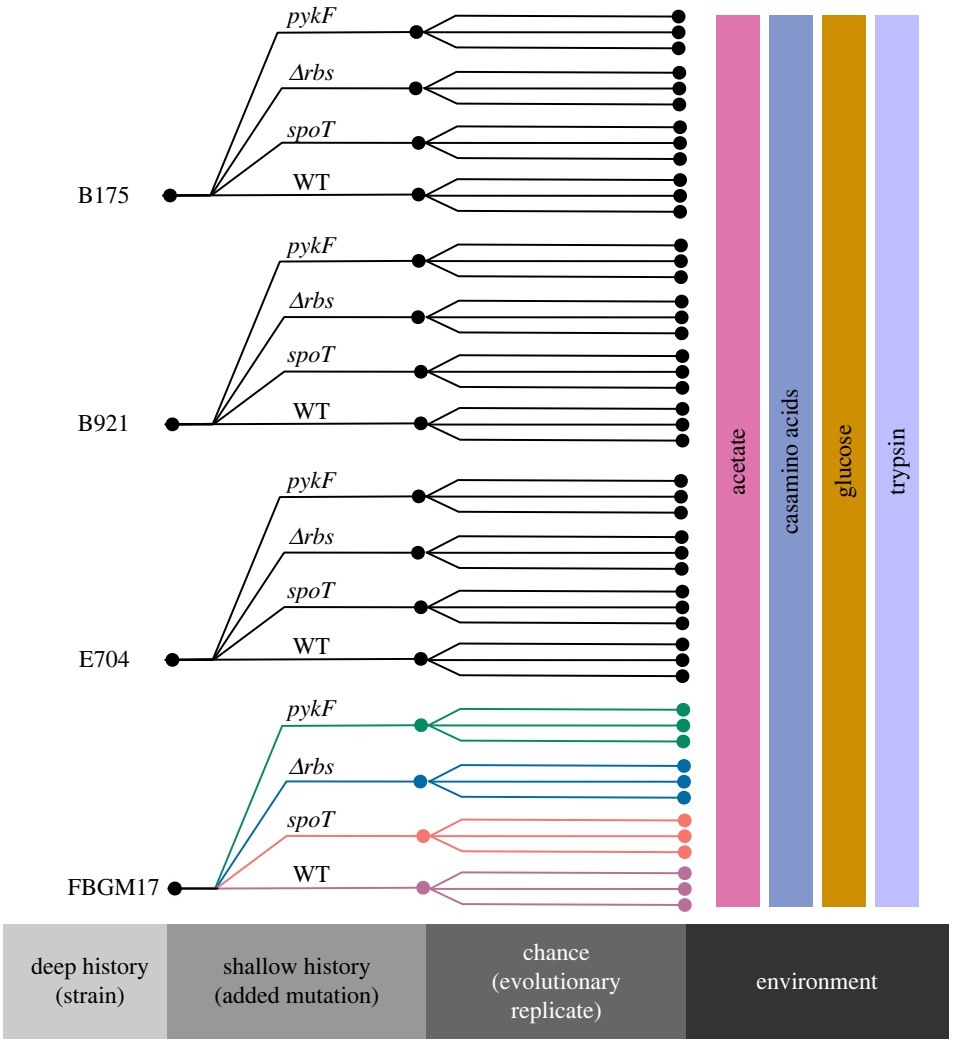

Figure 1. Schematic of evolved population organization. Four progenitor natural isolate strains of *E. coli* (deep history) were each divided into four sub-groups comprising the original strain (wild type (WT)) and three new strains with distinct added mutations that were selected in populations evolved as part of an earlier experiment (shallow history) [24,51]. Each of the 16 strains was used to found three replicate populations (chance) in each of four selection environments. Each population was evolved for 2000 generations and then fitness changes compared against its progenitor. (Online version in colour.)

(TA) indicator agar [51]. In competitions between reference Ara- and evolved Ara+ strains, samples were grown from frozen stocks for 24 h in lysogeny broth (LB) and then transferred to allow growth in their respective evolution resource environment for an additional 24 h. After this preconditioning, competitions were started by mixing equal volumes of an evolved population and its corresponding Ara- ancestor at a combined 1000-fold dilution in fresh media. A sample was immediately plated on TA agar to determine the initial frequency of each competitor. A second sample was plated following 24 h of competition. Malthusian parameters of each competitor were estimated as: $m = \ln(N_1/N_0)$, where N_0 and N_1 represent initial and final densities, respectively. The relative fitness of evolved populations was calculated as the ratio of Malthusian parameters of evolved and ancestral competitors. The effect of the Ara marker in each strain–resource combination, as determined by control competitions between Ara+ and Ara- ancestor strains, was subtracted from relevant fitness estimates. Competition assays were carried out in complete blocks with fourfold replication.

(d) Estimation of natural isolate strain fitness using growth curves

Competitive fitness assays allow fitness of derived or evolved strains to be compared to a corresponding progenitor strain. It is not possible to extend this approach to compare the fitness of our distinct progenitor strains because competition between these strains may be affected by complex interactions, for example, mediated by colicins or phage, that lead to non-transitive fitness relationships. To estimate fitness of all starting and evolved strains on a comparable scale, we analysed growth curves of progenitor strains to estimate their fitness relative to one another as determined only by differences in resource use [56]. In subsequent comparisons between progenitor strains, we chose E704 as the reference strain because it had the lowest mean fitness across the four assay environments. The fitness of progenitor strains estimated in each environment was added to competitive fitness estimates of evolved populations to obtain final fitness values for all evolved populations.

(e) Genetic and biology relatedness

Core and accessory genomes were estimated by aligning 250 base windows across a panel of 90 natural isolate *E. coli* strains using PANSEQ [57]. PhyML was used to build a maximum-likelihood tree of the core genome. For the accessory genome, a binary input file indicating the presence/absence of each accessory gene in each strain was analysed using default parameters of PARS in PHYLIP [58]. The diet profiles of the strains were measured using Biolog PM1 plates. These plates allow estimation of the respiratory activity of each strain on each of 95 distinct substrates (Biolog, Hayward, CA). A neighbour-joining tree was constructed

using Biolog data using the program Neighbor in PHYLIP [58]. To determine the fraction of common genes in the progenitor strains used in this study we annotated genomes using Prokka 1.14.6 [59] and compared them to identify core and accessory genes using Roary 3.11.2 with amino acid alignment [60].

(f) Statistical analysis

We used mixed-effects linear models to partition variation in final fitness and the difference between initial and final fitness (change in fitness) among the various factors in the experimental design. Statistical analyses were performed using R 4.1.0 [61]. Estimation of variance components was performed using the lmer function from the lme4 package [62]. CIs of variance components were calculated using the confint function of the VCA package. We tested for an effect of initial fitness as a covariate by fitting models with and without that term and comparing them using the anova function. In all cases, the fit of models was improved by the addition of an assay block effect and this effect was included in models reported here. History was assessed at the level of progenitor strain (deep history) and of added mutation (shallow history) by adding these as random effects and estimating their s.d. as the square root of their respective variance components. Reporting the s.d. gives values of these effects comparable to the mean change in fitness due to adaptation. Chance was estimated similarly as the s.d. of the variance component of replicate populations nested under each founder strain. Adaptation was reported as the grand mean fitness or fitness improvement of relevant evolved populations.

3. Results

(a) Influence of history, chance and environment on the fitness of evolved populations

We evolved four natural isolate strains and derivatives containing one of three additional mutations for 2000 generations in each of four selection environments (figure 1). After this evolution, we determined the relative influence of adaptation, deep (strain) and shallow (mutations added to each strain) history, the interaction between deep and shallow history and chance (variation among evolutionary replicates) on fitness (figure 2). Adaptation was significant in all environments, reflecting an increase in fitness at the end of the experiment. Deep history explained a significant amount of variation in fitness between strains at both the start and end of the experiment. Indeed, in the casamino acids and trypsin environments, deep history contributed more than adaptation to final population fitness. The effect of shallow history was initially small and changed little following evolution. Shallow history did, however, interact significantly with deep history in the casamino acids and glucose selection environments, indicating that introducing different engineered mutations resulted in different final fitness in different progenitor strain backgrounds. Finally, chance effects were generally small and not significant. Together, these results indicate a substantial influence of adaptation and an ongoing effect of deep history in determining population fitness after a period of lab evolution.

(b) Effect of history on fitness change

A complication of the above analysis is that initial fitness differences between progenitor strains are large relative to changes in fitness that occurred during the evolution experiment (electronic supplementary material, figure S1). For this reason, we repeated the analysis using fitness change relative to the relevant progenitor strain as the response variable, thereby removing the direct effect of initial fitness on determining final fitness. The importance of deep history was smaller in these models, though it remained significant in acetate, glucose and trypsin selection environments (figure 3). The effect of shallow history increased, becoming similar to that of deep history in the casamino acids and glucose environments, and interacting significantly with deep history in all but the acetate environment. Again, chance effects were generally small and not significant. These results indicate a dominance of adaptation in determining fitness changes but also that history continues to have a significant influence.

(c) Initial fitness of strains predicts shallow, but not deep, history effects on fitness change

Previous studies have found that initial fitness can influence rates of adaptation, and it is possible that some of the variation in evolved fitness changes can be explained by the different fitness of founder strains, rather than on specific effects of their genotypes [25,26,41,52,63,64]. In particular, many studies have found a pattern of diminishing returns, whereby fitter populations tend to have lower rates of adaptation than less fit populations [25,26,41,45]. To test this, we repeated the above analysis of fitness change explicitly including the initial fitness of each founder strain as a covariate. We found that initial fitness had a significant, or marginally significant, effect on fitness change only for populations evolved in the casamino acids and glucose environments (electronic supplementary material, table S1, figure S2). Counter to our expectation, however, this relationship was generally positive, such that strains with high initial fitness had higher fitness increases (Pearson correlations: acetate: $r = 0.13$, $p = 0.38$; casamino: $r = -0.34$, $p = 0.02$; glucose: $r = 0.27$, $p = 0.06$; trypsin: $r = 0.19$, $p = 0.20$; electronic supplementary material, figure S3).

To test if the generally weak relationship between initial fitness and changes in fitness also held among the much more closely related strains derived from each natural isolate, i.e. at the level of shallow history, we repeated the above analysis separately for each progenitor natural isolate strain. Here, we found strong support for a pattern of diminishing returns: more fit derivatives of each progenitor improved less than less fit derivatives (Pearson correlations were negative in 15/16 comparisons, binomial test $p < 0.001$; electronic supplementary material, figure S3, table S2). These results indicate that initial fitness can predict the potential for fitness change among closely related strains that differ in their shallow history, but not among more distantly related strains.

(d) Evolution environment contributes to fitness differences

To evaluate the dependence of selection environment on the influence of chance, adaptation and history effects, we considered a combined model that includes the contribution of the selection environment to fitness change (figure 4). We found that environment was a significant contributor to variation in fitness change of the evolved populations. The effect of deep history remained significant whereas shallow history did not.

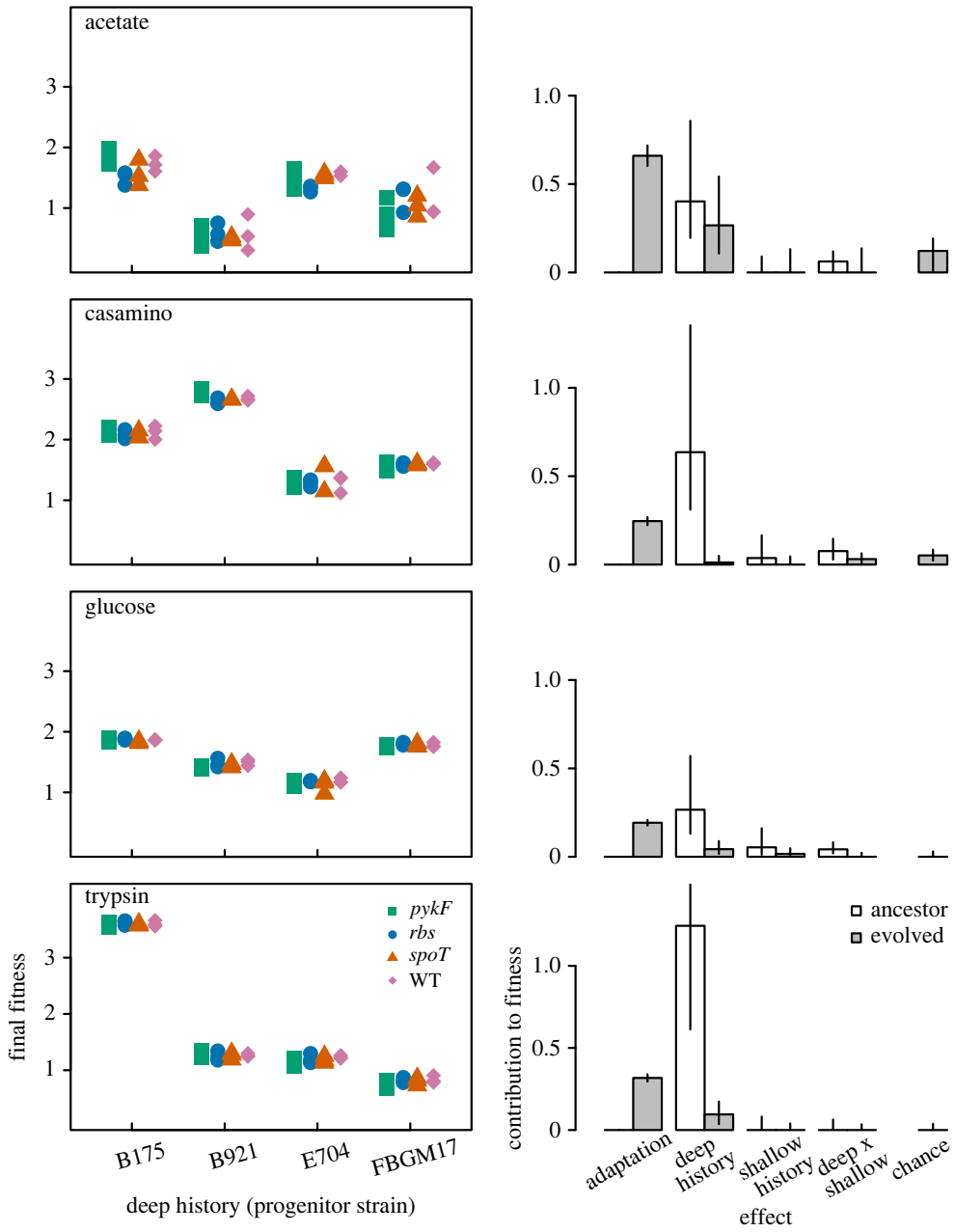

Figure 2. Contribution of adaptation, deep history, shallow history and chance to final fitness in each environment. Left-hand panels indicate the final fitness of replicate founder strain populations evolved in each environment. The contribution to fitness of adaptation, deep and shallow history, their interaction and chance, are indicated in the right-hand barplots. Contributions were estimated as the square root of the relevant variance components. Hollow and solid bars indicate ancestral versus evolved populations, respectively. Note we could not estimate effects of chance or adaptation for ancestral populations. Error bars indicate 95% CIs. All fitness estimates were performed with fourfold replication. (Online version in colour.)

(e) Influence of phylogeny on adaptation

It is possible that the phylogenetic relationships among strains predict the influence of deep history on evolutionary outcomes. Alternatively, the effect may be idiosyncratic, such that the effect of deep history varies unpredictably over the phylogeny. To test for a role of phylogenetic signal in determining responses we measured the influence of phylogenetic relationships inferred considering either the core or accessory genomes of progenitor strains on degree of fitness change. We also considered the predictive value of a dendrogram based on growth of progenitor strains in 94 distinct environments (Material and Methods, [65]). The null hypothesis in these tests is that a phenotypic trait will be distributed randomly over a phylogeny or dendrogram, that is, without phylogenetic signal. This hypothesis was rejected in only three instances (of 48 possible, given by four environments, four shallow histories and three phylogeny/dendrogram combinations). Notwithstanding the low statistical power of individual tests, this overall lack of support for any phylogenetic signal is consistent with fitness change not being predictable on the basis of either the phylogenetic relationship or initial ecological performance of progenitor strains (electronic supplementary material, table S3).

4. Discussion

We quantified the influence of chance, history, adaptation and environment on evolutionary outcomes by analysing evolved fitness changes of a series of populations founded from divergent natural isolate strains of *E. coli*. We found that differences in deep history were consistently reflected

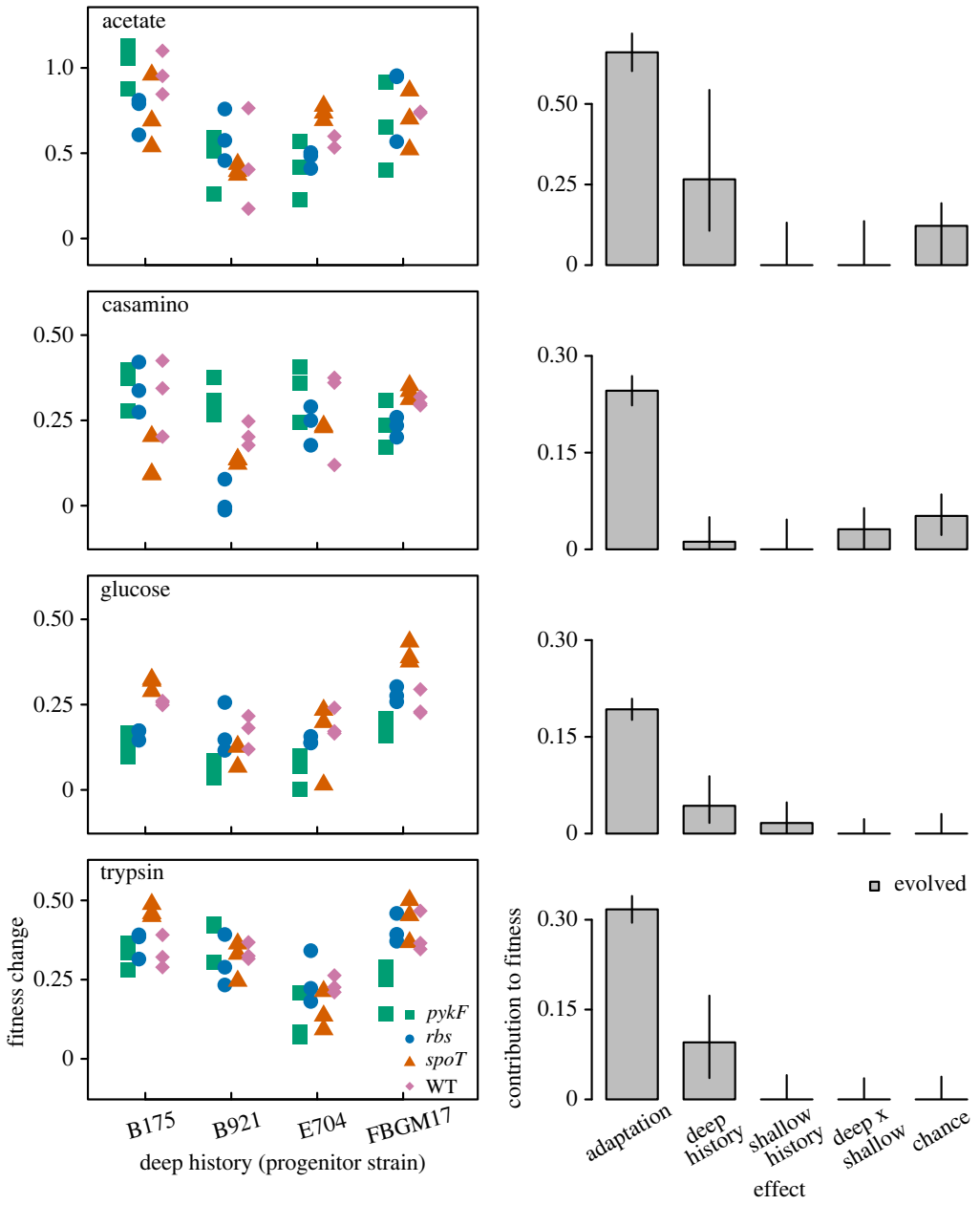

Figure 3. Contribution of deep history, shallow history and chance to change in fitness in each environment. Organization of plots are as for figure 2 except that effects are only considered for evolved populations because fitness change is not applicable to ancestral strains. Note different Y-axis scale for populations evolved in acetate. (Online version in colour.)

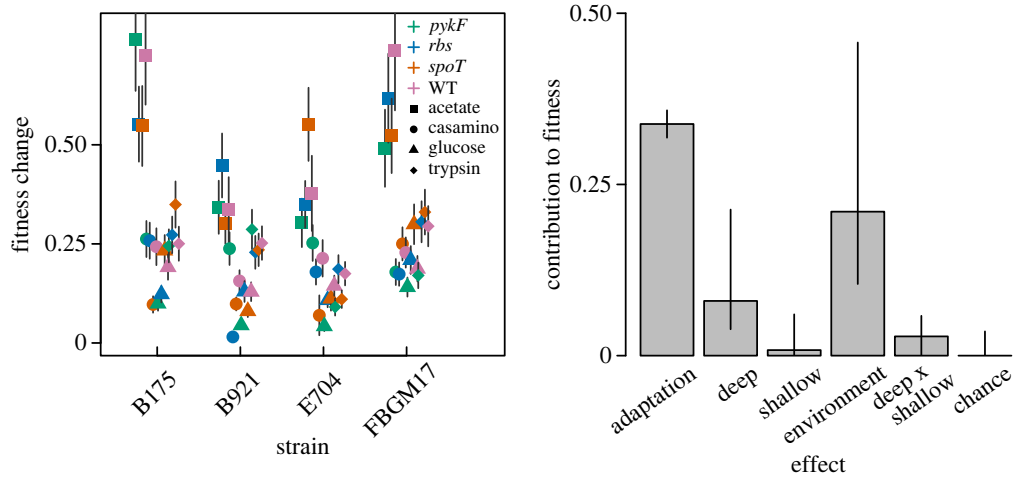

Figure 4. Dependence on environment of chance adaptation and history effects. Fitness change of evolved populations over all evolution environments. The contribution to fitness of deep and shallow history, evolution environment, the interaction between deep and shallow history, adaptation and chance, are indicated in the right-hand barplot. Error bars indicate 95% CIs.

in differences in fitness change and final fitness. By contrast, introduced mutations representing shallow historical differences had little effect on the final fitness of populations but did affect the pattern of fitness change. These findings were consistent across four tested evolution environments, though environment did affect the relative success of strains. Evidently, evolutionary outcomes were strongly dependent on combinations of history and environmental factors.

Several laboratory evolution experiments have used controlled and replicated treatments to measure the influence of chance and genetic history on evolutionary outcomes [37,42,43,46,51,66–68]. For example, Travisano *et al.*, [43] found that a period of selection in glucose caused fitness differences between populations that were reduced, but not erased, following a subsequent period of selection in maltose. Similar studies have found differences in fitness change between populations with histories of selection in different environments, including selection for antibiotic resistance mutations [14,36,40]. However, these studies have generally considered the effect of history between very closely related strains. By contrast, the four natural isolate strains that represented the deep history of our experimental design were very divergent, sharing as few as 57% of their genes (strains E704 and FBGM17 share 3485 genes as their core genome but have a combined 6059 genes in their pan genome) [52]. This divergence was reflected in large initial fitness differences between the founder strains and underlies deep history as the most important determinant of the final fitness reached by evolved populations (figure 2).

Deep history was also a significant contributor to differences in changes in fitness occurring during our experiment. It is notable, however, that shallow history explained as much variation in fitness change as deep history in the glucose environment and interacted with deep history in two other environments (figure 3). While the importance of shallow history in glucose may, in part, be due to the added mutations being originally selected for their fitness effects in that environment, its contribution to fitness change in other environments underlines how sensitive evolutionary outcomes can be to even small differences in the starting genotypes of populations. Given this interpretation, it is perhaps surprising that inevitable chance differences in the mutations substituted in replicate populations did not generally cause chance to be more influential in our experiment. One explanation is that mutations arising early in the adaptation of replicate populations to novel environments targeted similar pathways, limiting the opportunity for divergent interactions with subsequent mutations. If our experiment had been continued for longer we expect that chance would become increasingly influential relative to shallow history as genetic differences between replicates become large relative to those initially introduced.

There are two broad and non-exclusive explanations for how history might affect fitness increases occurring in our experiment. The effect and identity of at least some available beneficial mutations might be idiosyncratic, depending on the specific genotype defining different strains [11,69–71]. In essence, starting populations are at places on the fitness landscape with different available mutational paths to fitter phenotypes, creating phylogenetic inertia or constraint. Alternatively, different outcomes might depend on the effect of new mutations depending on some global phenotypic property of a strain, a mechanism known as macroscopic epistasis [72]. This possibility is supported by several studies finding that a significant portion of mutation effects can be explained by the starting fitness of a recipient strain independent of its specific genotype, following a pattern known as diminishing-returns epistasis [24–26,41,73,74].

We find evidence consistent with both explanations presented above, depending on the level of divergence between founding strains. Considering all 16 of the founder strains evolved in each environment, we found no clear effect of initial fitness on fitness change (electronic supplementary material, figure S2). This absence of overall pattern, however, hid a significant effect of decreasing returns at the level of shallow history. In all but one case (all but derivatives of B175 in the acetate environment) derivatives of a progenitor natural isolate strain with higher initial fitness achieved smaller subsequent fitness increases (electronic supplementary material, table S2). This pattern is consistent with the mechanism of diminishing-returns epistasis, though we note that the same pattern might occur through specific mutation interactions if antagonistic interactions become systematically more likely in higher fitness genotypes [69–71]. In any case, the different effect of initial fitness on subsequent fitness changes seen at the two levels of history indicates that opportunities for fitness improvement are not consistent across different levels of strain divergence, even during adaptation to the same environment.

A possible explanation for differences in the pattern of diminishing returns at deep and shallow levels of history is that it depends on the distance of starting populations to a fitness peak [69,75,76]. These distances vary much more at the level of deep compared to shallow history. This effect, however, predicts higher fitness increases in populations founded by low fitness natural isolate strains and is, therefore, expected to reinforce an overall pattern of diminishing returns, which was not seen. An alternative possibility is that populations started by low fitness natural isolate strains were far enough from fitness peaks that many beneficial mutations could occur and, because populations were asexual, interfere with each other's fixation [77]. This interference can impose a 'speed limit' to adaptation, but again, does not explain lower fitness populations having smaller fitness increases. Whatever the explanation for different levels of history having different influence on diminishing returns it has evolutionary consequences, implying that predictability of evolutionary responses might be limited to closely related genotypes.

Finally, we emphasize that our focus here was on factors affecting changes in the fitness of evolving populations. Of course, similar fitness outcomes can result from distinct underlying phenotypic changes caused by different genetic changes [78]. This many-to-one mapping means that the influence of the factors we consider might differ when assessed at different biological levels, e.g. changes in genotype or in lower-level phenotypes such as the specific metabolic and physiological changes underlying fitness improvement. Specifically, evolutionary outcomes assessed at the level of fitness are likely to be more convergent, and less contingent, than outcomes assessed using lower-level phenotypes or genotypes [26,37,79]. For this reason, our finding of consistently significant effects of history is likely to be conservative with respect to its effects acting at lower levels of biological organization.

In summary, we find that adaptation to the selective environment, deep genetic history and distinct shallow histories can all affect the evolution of a population. A consequence of history contributing to evolutionary outcomes, especially as it

interacts with environment, is that evolution will often be unpredictable. Evidently, even small genetic differences between populations can change either the availability or benefit of specific evolutionary paths [80].

Data accessibility. Data and analysis scripts are available from the Dryad Digital Repository (https://doi.org/10.5061/dryad.4f4qrfjfs) [81].

Electronic supplementary material is available online [82].

Authors' contributions. C.E.S.: conceptualization, formal analysis, investigation, methodology, writing—original draft; A.N.H.S.: formal analysis; T.C.: conceptualization, formal analysis, funding acquisition, writing—review and editing; F.M.: conceptualization, formal analysis, funding acquisition, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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