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The Pleiotropic Effects of Beneficial Mutations of Adapted Escherichia coli Populations

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Abstract:

Mutations that improve fitness in one environment can often be beneficial, deleterious, or neutral in alternative environments. When a single mutation effects fitness in multiple environments, it is said to be a pleiotropic, which can have important consequences for niche specialization, niche expansion, speciation, and even extinction in the face of environmental change. While previous studies have revealed that pleiotropy is nearly universal, the role of adaptive history in the spectrum of pleiotropic effects has yet to undergo detailed experimental observation. Using experimental evolution we gathered beneficial mutations in a previously adapted strain of *Escherichia coli* growing in the same common substrate over hundreds of generations. We then tested the effects of these mutations in multiple alternative environments and compared their fitness to the ancestor. We found that the magnitude of the effects correlates positively with the similarity of resources to glucose, indicating that selective history has an influence in the distribution of beneficial mutations. These findings broaden our understanding of the effects of history on pleiotropy, and may provide answers into how evolution in a constant environment influences ecological niche formation and constraint.

Introduction:

Beneficial mutations with multiple (or pleiotropic) effects in alternative environments are not clearly understood, despite the fact that a broad research base suggests that it is nearly universal (Ostrowski et al, 2005). The challenges of predicting such mutations and their consequences in a range of selective environments are numerous. Moreover, the data that does exist has led to contradicting modes of thought, with many declaring the relationship to be primarily antagonistic (citation here), while others find evidence that beneficial mutations typically generate positive pleiotropy (Otto 2004; Ostrowski et al, 2008).

Depending on the direction of pleiotropy, the ramifications for ecological stratification and speciation range widely. Positive pleiotropy would most likely orient a population towards the development of a broad ecological niche, known as a niche-generalist, whereas antagonism would likely result in a niche-specialist. In addition, the adaptive history and the methods of mutation collection or assessment inherent in the generation of pleiotropic mutations, particularly in long-term evolutions, may modify subsequent adaptive outcomes, further complicating interpretations of pleiotropic effects (Leiby and Marx, 2014).

The question of adaptive history influencing the distribution and effect of mutations can be ideally answered by bringing evolution into the lab and relatively quickly playing out its traditionally slow march under controlled conditions. Previous studies have already touched upon the effects of history on adaptation in *Escherichia coli*, with prior growth in glucose resulting in fitness variability in maltose (Travisano et al, 1995) and long-term growth in a minimal glucose environment caused wide-spread reduction in resource catabolism (Cooper and Lenski 2000). However, these developments did not identify the nature (beneficial, neutral, or deleterious) of the mutations that garnered the observed effects: the experimental evolutionary

model used by the abovementioned sources was used to generate mutations of unknown quality and quantity, as did Ostrowski et al in their assay of alternative environments (Ostrowski et al, 2005). By strictly gathering single, beneficial mutations with smaller, total fitness effects within pre-adapted populations over a short term-evolution, the effects of these rare mutations can be more accurately parsed out in alternative environments.

Bacterial populations are ideal candidates for experimental evolution because they have low generation times and large population sizes allowing for rapid mutation collection during an evolution. Ease of lab-based culture techniques and non-growing storage at -80°C also allows for reliable comparisons between evolved populations and the ancestor, a crucial consideration for mutation analysis. *E. coli* is an obvious choice for use in answering a question such as this, with a long history of being harnessed in the field of evolutionary biology, most notably in Richard Lenski's Long-Term Experimental Evolution (LTEE) project (Lenski, 1990). More specifically, *E. coli* B REL 1206 (henceforth REL 1206) is an optimal choice for goals chosen for this study. REL 1206 is an evolved version of the progenitor of *E. coli* strain REL 606 used to begin the LTEE, with two thousand generations and five mutations already accumulated prior to this study. Strain REL606 is notable in that it has been completely purged of plasmids and bacteriophages, preventing any sharing of genetic information between individuals in a population. This effectively removes the possibility of sexual recombination from the scope of the experiment.

Pleiotropic effects can be assessed in any number of selective, alternative environments, ranging from temperature to catabolic substrates. By analyzing the beneficial mutations acquired in an evolution in a common substrate, such as glucose, against a panel of alternative sugar environments, the patterns of pleiotropy can be observed. It is likely that growth in a single, selective environment for an extended period will generate beneficial mutations that will drive

adaptation towards specialization, rather than generalization, and that antagonistic pleiotropy will be more prominent in populations exposed to substrates dissimilar to the original, common substrate. Here, we explored the pleiotropic effects of glucose-adapted mutants in four environments after generating individual, beneficial mutations in a short-term evolution. We predict that evolution in a single catabolic substrate will generate mutations with beneficial, direct effects in that environment, but will have increasing antagonistic effects as the relatedness to glucose decreases.

Materials and Methods

Experimental Evolution

This study requires the steady growth of bacterial populations in a constant environment in order to quickly produce isolated, beneficial mutations for later analysis in alternative environments. The progenitor for the experimental populations used in this study was *E. coli* REL 1206, a clone isolated following two thousand generations of evolution in glucose at 37°C, which had concurrently acquired five separate mutations (Lenski 1991). This progenitor was modified for the purposes of this evolution and following fitness assays to express different, fluorescent markers: Yellow Fluorescent Protein or Cyan Fluorescent Protein (CFP or YFP). *E. coli* marked with YFP experience a positive fitness effect of approximately 1%, requiring correction factors for proper analysis. Each variant, hereafter known as REL 1206 CFP and 1206 YFP, was isolated from -80°C frozen culture on Luria Broth media plates and a single colony picked from each isolated variant to serve as the ancestor for the opposing, marked lines.

Before beginning the evolution, the isolated 1206 CFP and YFP colonies were grown in 5 mL Luria Broth at 37°C in 13x100 mm glass culture tubes rotating in a roller drum. After 24 hrs.

50 μL of each culture was diluted in Phosphate Buffered Saline and pipetted into 18 13x100 mm glass culture tubes (36 tubes total) with 5 mL of Davis Minimal Media (25 μL/mL of glucose). Twelve of the DM replicates received culture diluted 10^{-2} in PBS while 6 replicates received culture diluted 10^{-4}. After 24 hrs. of growth at 37°C, 25 μL of each culture was added to 5 mL of DM25 in 13x100 mm glass culture tubes, for a total of 18 tubes with equal parts 1206 CFP and YFP, with the corresponding dilutions. Tubes were vortexed at medium agitation for twice for 1 sec before culture was pipetted from the previous day's culture tubes, after culture had been pipetted into dilution tubes, and again after culture had been added to fresh culture tubes. The populations were transferred on a 24 hr. basis for a total of 61 days.. Controls of purely CFP and YFP cultures were maintained and transferred in parallel to the evolving populations. Approximately every 10 days, 1.47 mL of each population culture was mixed with 0.13 mL of glycerol and stored at -80°C.

Tracking Relative Frequency

To determine the nature of the shifting ratio of the oppositely marked *E. coli* growing in co-culture, the populations were diluted and submitted to high-throughput sampling. On Day 0 and every 3-4 days until the termination of the evolution, the frequency of the evolving population was measured via flow cytometry utilizing a Guave easy-Cyte 8HT flow cytometer and InCyte software analysis (Millipore Inc.). Pertinent setting configurations are as follows: Forward Scatter Gain-79.48; Side Scatter- 4.97; Green Laser- 304.44; Threshold- 33; Blue Laser- Active; Flow Rate- 0.24 μL/sec. Two hundred microliters of each co-culture and controls were pipetted into a 96-well plate, with a blank and calibrating controls of YFP and CFP cultures included. The cultures were diluted to $10^{-2\frac{1}{2}}$ in filter sterilized PBS, then submitted to flow

cytometry analysis. Five thousand events were read from each replicate, with cell concentrations typically in the range of 300-400 cells/µL.

Beneficial Mutant Isolation

To isolate suspected single, beneficial mutations, variations in the trend of YFP:CFP frequencies were observed and pure colonies picked and then frozen. To accomplish this isolation, the co-cultures of samples featuring an observable change in the population ratio for two contiguous days were quadrant streaked on Davis Minimal Media glucose (25µg/mL) plates. After growing at 37°C for 24 hrs. approximately half of eight random colonies were picked and diluted 10^{-4} in PBS, and then submitted to flow cytometry analysis. The colony corresponding to the appropriate marker were inoculated in 5 mL Luria Broth in 13x100 mm glass tubes and grown in a roller drum at 37°C for 24 hrs. After incubation, 1470 µL of culture was mixed with 130 µL glycerol and stored at -80°C. After colonies had been isolated, the population was either stopped or continued for another 7-10 days, when then the isolation procedure repeated, in cases where the population shift was less substantial.

Fitness Assays

To compare the relative fitness of the acquired beneficial mutants to that of the ancestor in glucose and in multiple, alternative environments, fitness assays were conducted on the gathered mutants. Frozen mutants were inoculated in 5 mL Luria Broth and grown in roller drums at 37°C for 24 hrs. Fifty microliters of culture were then pipetted into 5 mL PBS (10^{-2} dilution) and then 50 µL diluent was pipetted into 5 mL Davis Minimal Media containing 25 µg/mL of substrate (glucose, maltose, n-acetylglucosamine, or lactose), and then grown for 24 hrs. at 37°C. After incubation, four replicates of 25 µL of mutant and 25 µl of oppositely marked

ancestor were combined in 5 mL DM substrate and incubated, while 100 μL each were pipetted into a 96-well plate (with blank and pure marker culture controls) for a Day 0 initial flow cytometry analysis, as described above. Fifty microliters of culture were transferred on Day 1 and Day 2, with a final flow cytometry analysis (200 μL of co-culture, this time) on Day 3.

Statistical Analysis

Data collected after flow cytometer assays was organized via Millipore's proprietary InCyte software onboard the flow cytometer. All data analysis was achieved using Microsoft Excel's spreadsheet and charting processes. Relative fitness values for the evolved mutants vs. the ancestor were calculated using $W = \ln w_f d0 \times 10^2 w_i \ln n_f (d3 \times 10^2) n_i$ where W is the fitness of the evolved mutant relative to the ancestor; w and n is the frequency of the mutant and ancestor, respectively; and i and f are initial (D0) and final (D3) frequencies respective to the mutant or ancestor (Lenski et al, 1991).

Data Corrections

Frequency corrections for the marked lineages versus the ancestor were completed using the equations $CF_B = B_f - ((A_f - B_f \times B_{CF}) \times A_{CF}) + ((B_f - (A_f \times A_{CF})) \times B_f)$ and $CF_A = A_f - ((B_f - A_f \times A_{CF}) \times B_{CF}) + ((A_f - (B_f \times B_{CF})) \times A_f)$ where $CF_{A/B}$ is the corrected frequency for the marked, beneficial mutant /ancestral competitor; B_f is the mutant frequency; A_f is the ancestral frequency; B_{CF} is the correction factor for beneficial mutant frequencies; A_{CF} is the correction factor for the ancestral frequencies. Correction factors were found for CFP ($YFP\ Freq. CFP\ Freq.$) and   YFP ($CFP\ Freq YFP\ Freq.$).

Results

Experimental Evolution-derived Beneficial Mutations

A total of eighteen lineages of *E. coli* REL 1206 generated nine distinct disturbances in the marked populations, after sixty one days and up to eight hundred generations of growth. Consistent decreases in YFP frequency during flow cytometry analysis was an indicator for the presence of a beneficial mutation appearing in the oppositely marked CFP population and rising to fixation. Figure 1 shows the growth curves of the low N_e populations, with mutants isolated at 450 and 650-725 generations. Figure 2 depicts similar growth curves for some of the high N_e populations (data not shown for isolated mutants). The populations evolved in Figure 2 showed no decline in YFP frequency until the last fifty generations or so, where all twelve lineages began to decline in parallel. High N_e mutants experienced noticeable YFP decline during their evolution.

Four of these mutants were derived from the low N_e replicates, while the other five mutants were derived from high N_e replicates. Of these nine confirmed mutants, one mutant gathered from a high N_e lineage was discarded from further experimentation after fitness assays (discussed below) in glucose and maltose achieved relative fitness effects that were identical to the control. This left a collection of eight working beneficial mutations for further competition and analysis in alternative environments.

Fitness Effects in Glucose

After the completion of the experimental evolution, the collected beneficial mutants were competed against the ancestral REL1206 strain in the historical glucose environment. Figure 3 shows the distribution of fitness effects in the adaptive glucose environment, as well as the three

alternative substrates. Direct fitness effects in glucose ranged from 0 to ~2.5%, with total sample effects tightly clustered together (each sample deviated within ≤ 1%).

Fitness Effects in Alternative Environments

After fitness effects were analyzed in the adaptive environment, identical analyses were carried out in three alternative substrates: N-acetyl-d-glucosamine, maltose, and lactose. Competition in NAG yielded a slightly more distributed range of effects, achieving a maximum 3% fitness effect, with all mutants featuring greater fitness than the ancestor. Alternatively, lineages grown in maltose experienced both positive and negative fitness effects. Four of the eight lineages featured positive fitness effects, maximizing with 1206CFP14 at ~5%, while two of the lineages had evidence of negative effects, at most showing a 2% decline in fitness. Two of the remaining mutants had non-significant effects in maltose.

The fitness effects observed in lactose were similar to those noted in maltose, in that effects were distributed both positively and negatively: the magnitude and distribution of those effects, however, were much more sizeable. Beneficial mutants competed against their ancestor in lactose achieved a 44% spread of effect, measured from the most negative to the most positive. Four of the eight mutants produced significant, positive fitness effects, with the highest, 1206CFP14, 22% above the ancestral baseline. Conversely, the other four mutants produced significant negative fitness effects, with 1206YFP7 (the most negative mutant fitness detected) at 22% below the ancestral standard. Given the separation between the maximal and minimal data points in the lactose competition, discerning the extent to which these extreme data-points are actually observed effects or the result of error. However, taking these two out-sized values out still leaves large fitness effects: 1206CFP13 and 1206YFP14 have fitness effects of 7% above

and ~6.5% below the fitness ancestor, respectively, which are still the largest effects surveyed in any of the four separate competitions.

Discussion

This study sought to bring a deeper level of understanding to the behavior of beneficial mutations collected in pre-adapted populations of *E. coli*. Pre-adapted *E. coli* lineages were evolved for up to eight hundred generations in a selective glucose environment to generate qualitatively beneficial mutations. These mutants were then competed against the pre-evolution ancestor in several alternative substrates to identify the direction and magnitude of their pleiotropic effects. Due to the growth history in glucose, mutations derived from REL 1206 lineages after continued evolution in the same substrate were expected to lead towards antagonistic pleiotropy in non-glucose environments. Prior studies have identified both specialization and tendencies towards generalist behaviors in evolved *E. coli* populations, the story remains in complete.

The data collected suggests, with a small degree of variation, that beneficial mutations acquired after evolution in a glucose medium bestow generally positive fitness effects in alternative environments (Fig. 4). All mutants in N-acetyl-d-glucosamine, most mutants tested in maltose and half the mutants grown in lactose were significantly more fit than their ancestral competitors. Most mutants exhibiting positive fitness effects averaged 1-3% better than the ancestor, with some exceptions reaching spanning 4-7% increases in fitness (three mutants grown in lactose and two maltose-grown mutants).

NAG was the only substrate that every mutant surveyed experienced significantly higher relative fitness, averaging ~1% better than the ancestor. NAG is structurally the least similar to

glucose of all the observed carbohydrates. NAG is an amide formation of glucosamine and acetic acid, and is a major constituent of bacterial and fungal cell walls. The other two substrates are simple dimers of common sugars: maltose is composed of a glucose dimer while lactose is a glucose-galactose unit. With that in mind, prior studies have found NAG, among other sugars that also utilize the phosphoenol phosphatase-phosphotransferase (PEP-PTS) translocation system, to have positive fitness effects when used as a growth medium for glucose-derived mutations (Ostrowski, 2005; Travisano and Lenski, 2000).

Additionally, NAG is taken into the cytosol via the phosphate transferase system and it is here that mutations would most likely have the largest effect. Mutations affecting the rate or efficiency of glucose uptake via the PTS pathway may have indirect effects on other PTS-based sugars. Glucose and NAG actually share the same PTS pathway, transported into the cytosol from the periplasmic space by PEP-PTS enzyme II. Moreover, maltose is transported across the inner membrane by the malX permease system, which shares ~35% of its amino acid sequence with the glucose and NAG EII enzyme, indicating a strong likelihood for pleiotropic effects (Reidl and Boos, 1991). Due to the overall interconnectedness of the PTS and non-PTS regulatory systems, mutations collected in a glucose background have a strong potential for alteration of non-PTS expression and function (Travisano and Lenski, 2000).

Examples of negative fitness effects were most observed in lactose-competed mutants, specifically 1206CFP14 and 1206YFP7 through 14. These mutants generated the most negative fitness effects in the entirety of the experiment with one mutant causing a 22% decline in fitness compared to the ancestor, and others decreasing fitness by 8-10%. All other mutants grown in lactose exhibited high levels of positive pleiotropy, much higher than the other substrates. Lactose is the most different of the assayed substrates, transported from the periplasm to the

cytosol via lactose permease, the second gene (*lacY*) on the *lac* operon, where the dimer is hydrolyzed to galactose and glucose by β-galactosidase (Abramson et al, 2003). Lactose metabolism is completely separate from the other three substrates until the dimer's hydrolysis. However, galactose utilization requires a complex conversion to glucose using three separate proteins, known as the Leloir pathway, and a mutation directly affecting PTS-related sugars wouldn't be pinpointed until tested in a distinct lactose environment (Frey 1996).

These large-magnitude effects observed in lactose are similar to observations made by Ostrowski et al during related glucose evolution and competitions in five alternative environments: lactose most resembled the competition in melibiose, which experienced highly antagonistic effects, with most replicates seeing up to a 35% loss in fitness, and at most 10% gains in fitness over the ancestor. These beneficial mutations acquired in a pre-adapted background, when grown in lactose, seem to donate fitness effects on a level similar to mutations gathered in a naïve genotype (REL 1206 vs. REL 606), although the both positive and negative distribution in the pre-adapted lactose competitions are greater than the naïve competitions observed in other studies (Ostrowski et al, 2005).

Future studies involving these collected mutations should continue further competitions in more carbon substrates, and should contain examples of non-sugar substrates to ascertain as wide a variety of environmental effects as possible. Also, matched environment comparisons of pre-adapted and the naïve genotypes should be executed so as to determine the differences in indirect effects. Furthermore, to understand the genetic basis of the beneficial mutations gathered in these pre-adapted lineages, these mutations must be detected, identified, and their actual expression determined. To do this, Illumina re-sequencing of the mutant lines and subsequent data analysis will identify possible mutation sites against the *E. coli* B REL 1206 reference

genome, and then confirmed by Sanger re-sequencing. A lower cost alternative to genetic re-sequencing is vectorette PCR, which would be especially useful for verifying insertion mutations, as these are the best documented mutation class with fitness effects (Schneider and Lenksi, 2004; Cooper et al, 2001). Given their documented effects in alternative environments, pinpointing these beneficial mutations in genes responsible for the translocation of sugars across the inner and outer membranes, and their regulatory mechanisms, is highly likely.

The pattern of pleiotropic effects associated with beneficial mutations does not appear to be directed towards antagonistic relationships in alternative environments. Rather, the indirect effects of beneficial mutations gathered in a pre-adapted genotype, generally trend towards positive pleiotropic mechanisms, and that these mutations are loosely dependent on th e similarity of the alternative environment to that upon which the mutations were collected. The effects of pleiotropy remain difficult to determine, and even more difficult to predict. The findings brought forward by these evolutions and competitions curiously do not seem to support the prior assumption that adaptation to a single selective environment would induce a trend towards specialization over generalization in evolved lineages. These are curious results for an ongoing investigation that promises to provide more answers to the pleiotropic question.

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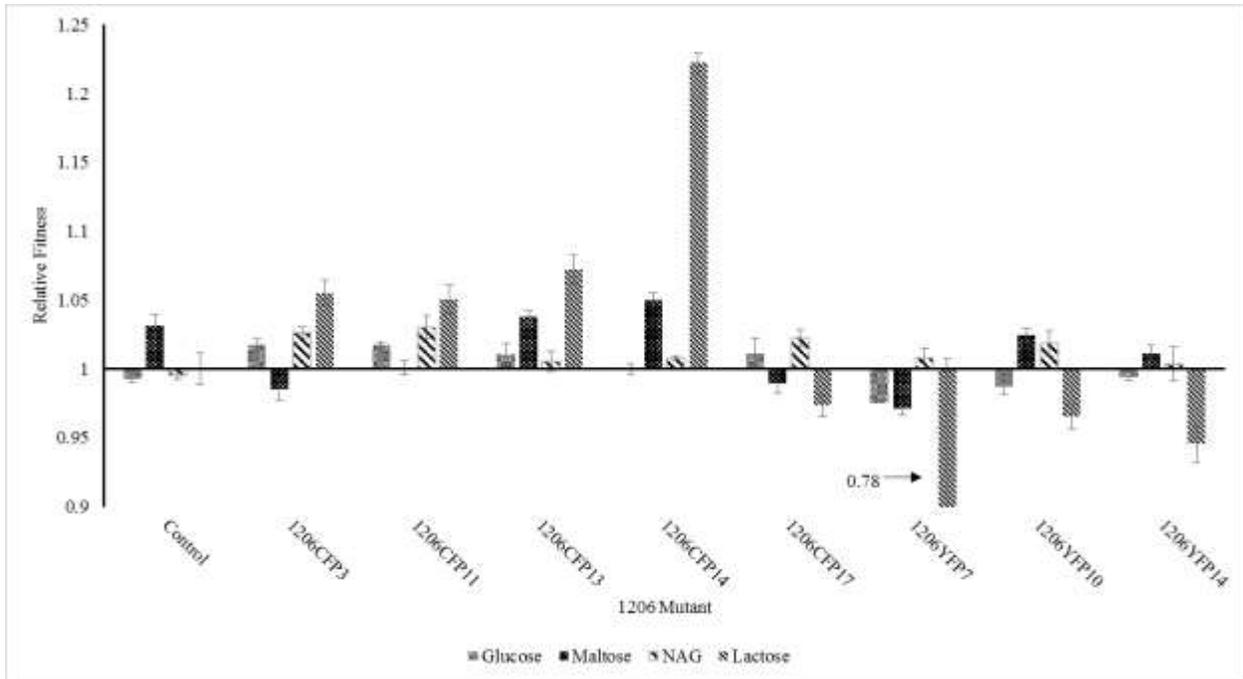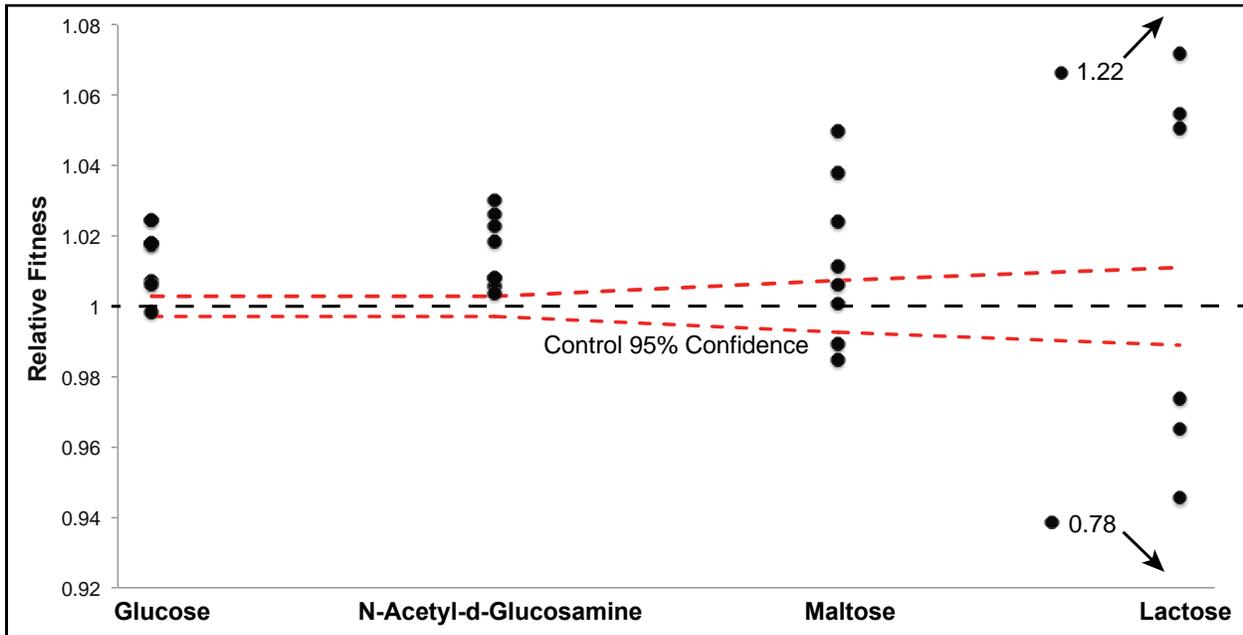
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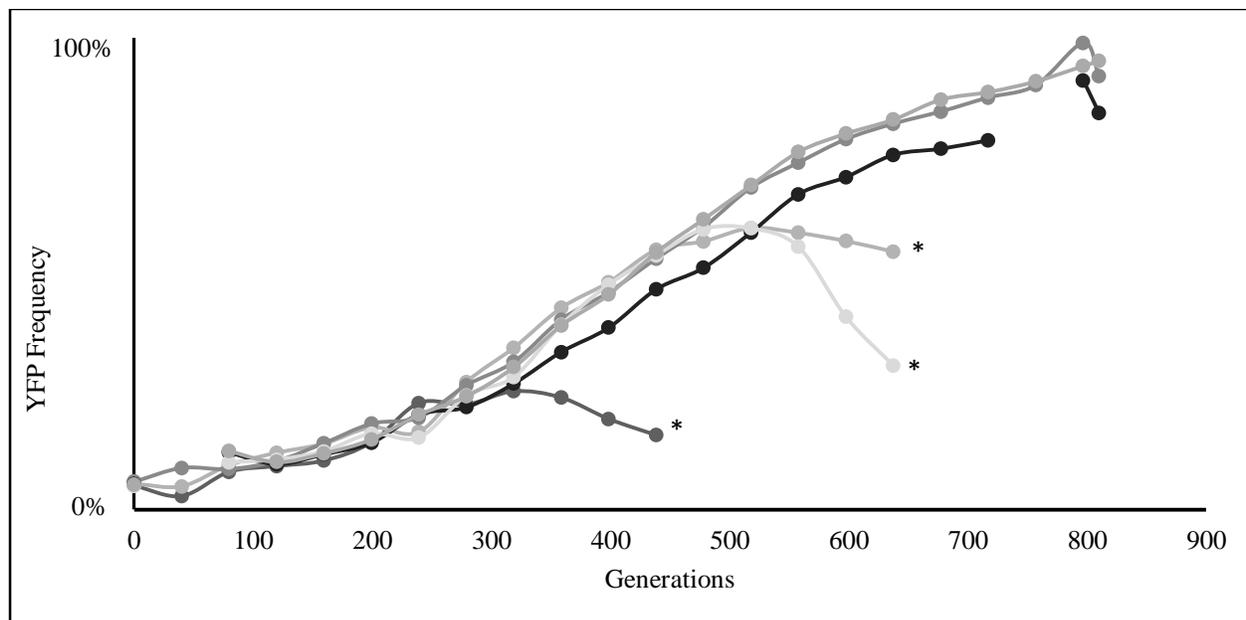
Figure 1: Low N_e experimental evolution frequency curve featuring YFP ratios over eight hundred generations. YFP frequency is the ratio of YFP-marked REL 1206 cells growing in relation to CFP-marked REL 1206 cells. The six shaded curves represent the separate, low N_e populations. Asterisked curves represent lineages selected for competitions in alternative environments: REL 1206CFP13, REL 1206CFP14, and REL 1206CFP17.
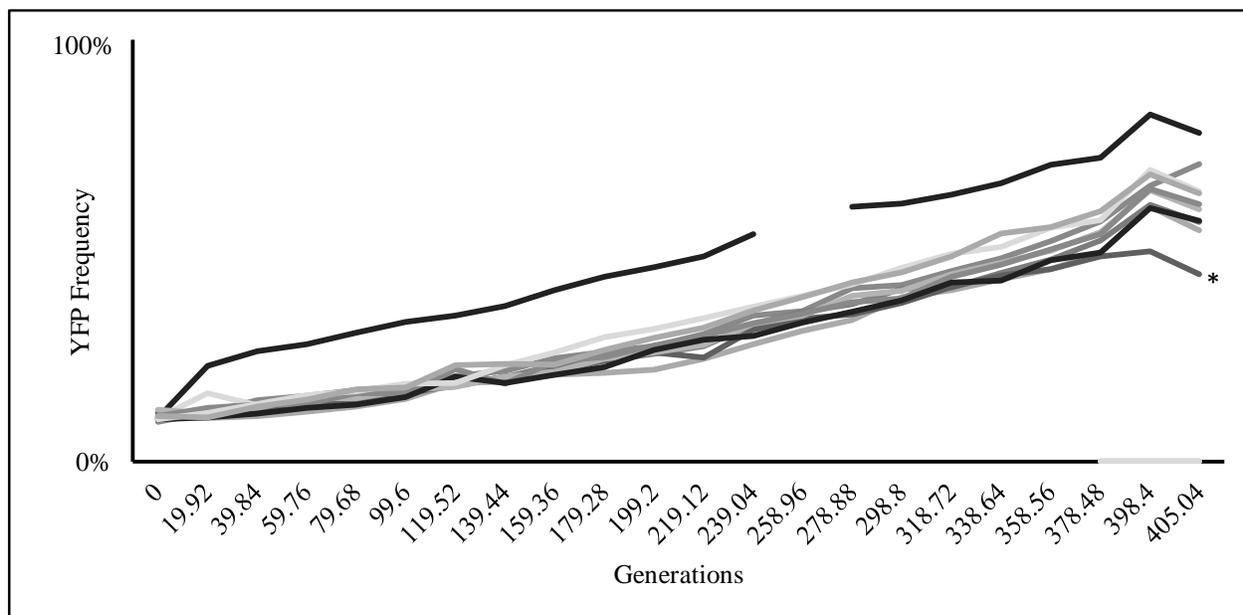
Figure 2: High N_e experimental evolution growth curve featuring YFP ratios over four hundred generations. YFP frequency is the ratio of YFP-marked REL 1206 cells growing in relation to CFP-marked REL 1206 cells. The twelve shaded curves represent the separate, high N_e populations. The asterisked curve represents REL 1206YFP7, which was selected for competition in alternative environments.

Figure 3: Average relative fitness effects of evolved REL 1206 beneficial mutants after competitions in a familiar glucose and three alternative environments: N-acetyl-d-glucosamine (NAG), maltose, and lactose. Relative fitness of the beneficial mutants to the ancestor calculated via $W = \ln wfd0 \times 102wi \ln nf (d3 \times 102)ni$ where W is the fitness of the evolved mutant relative to the ancestor; w and n is the frequency of the mutant and ancestor, respectively; and i and f are initial (D0) and final (D3) frequencies respective to the mutant or ancestor (Lenski et al, 1991). Each environment data point is a separate lineage made up of the average of four replicates, with the control for each represented by the dashed line, with 95% confidence calculated for $n=32$.

Figure 4: Distribution of the pleiotropic effects of beneficial mutants in four catabolic substrates: glucose, maltose, n-acetyl-d-glucosamine (NAG), and lactose. Columns represent averages of four replicates per mutant.