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Diverse phenotypic and genetic responses to short-term selection in evolving *Escherichia coli* populations

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Beneficial mutations fuel adaptation by altering phenotypes that enhance the fit of organisms to their environment. However, the phenotypic effects of mutations often depend on ecological context, making the distribution of effects across multiple environments essential to understanding the true nature of beneficial mutations. Studies that address both the genetic basis and ecological consequences of adaptive mutations remain rare. Here, we characterize the direct and pleiotropic fitness effects of a collection of 21 first-step beneficial mutants derived from naïve and adapted genotypes used in a long-term experimental evolution of *Escherichia coli*. Whole-genome sequencing was able to identify the majority of beneficial mutations. In contrast to previous studies, we find diverse fitness effects of mutations selected in a simple environment and few cases of genetic parallelism. The pleiotropic effects of these mutations were predominantly positive but some mutants were highly antagonistic in alternative environments. Further, the fitness effects of mutations derived from the adapted genotypes were dramatically reduced in nearly all environments. These findings suggest that many beneficial variants are accessible from a single point on the fitness landscape, and the fixation of alternative beneficial mutations may have dramatic consequences for niche breadth reduction via metabolic erosion.

KEY WORDS: Adaptation, adaptive history, beneficial mutations, niche breadth, pleiotropy.

Adaptation proceeds through the accumulation of beneficial mutations that provide an advantage in the selective environment. The magnitude of the fitness effects provided by beneficial mutations typically declines as organisms adapt (Chou et al. 2011; Khan et al. 2011; Rokyta et al. 2011; Good et al. 2012; Wiser et al. 2013; Kryazhimskiy et al. 2014; Nahum et al. 2015), but how adaptation changes the shape of the distribution of the fitness benefits is less certain (Kassen and Bataillon 2006; Martin and Lenormand 2008; Good et al. 2012). In addition, the molecular targets of beneficial mutations appear to be relatively limited

in the early steps of adaptation (Travisano et al. 1995b; Woods et al. 2006; Ostrowski et al. 2008), but may vary as evolution proceeds (Barrick et al. 2009). Both the direct (selected) and indirect (pleiotropic) effects of beneficial mutations are of central importance to countless biological processes, including adaptation (Cooper 2002; Leiby and Marx 2014), speciation (Cooper and Lenski 2000; Otto 2004), senescence (Holt 1996), antibiotic resistance (Kassen and Bataillon 2006; Bataillon et al. 2011), and the emergence of new pathogens (Couce and Rodríguez 2015). Therefore, characterizing the direct and pleiotropic effects of beneficial

mutations as organisms adapt, along with their molecular targets, is fundamental to our understanding of adaptive evolution (Nahum et al. 2015).

While all aspects of the nature of beneficial mutations require further study, the environmental dependence of both the magnitude and sign of fitness effects has received especially little consideration, despite evidence that pleiotropy is abundant and possibly even universal (Cooper and Lenski 2000; Cooper et al. 2001; Rozen et al. 2002; Dudley et al. 2005; Ostrowski et al. 2005; Kassen and Bataillon 2006). Historical models of adaptation have often incorporated pleiotropy, but make several assumptions about the nature of pleiotropy that lack experimental support. Many theoretical models assume that pleiotropy is a largely antagonistic process, and that large-effect mutations are more predisposed to antagonistic pleiotropy than small-effect mutations (Fisher 1930; Lande 1983; Orr and Coyne 1992; Otto 2004). This framework has been invoked to explain the evolutionary success of small effect mutations, the variability of evolutionary trajectories, and why we see such immense diversity in nature despite the phenotypic parallelism observed in replicate laboratory populations (Travisano and Lenski 1996; Cooper et al. 2003; Ostrowski et al. 2008). However, too few data exist on the distribution of pleiotropic effects to assess the validity of these assumptions under conditions that vary in their environmental heterogeneity, population sizes, or mutation rates (Good et al. 2012). A more complete understanding of the nature of beneficial mutations and their pleiotropic effects would benefit many fields of research.

The long-term evolution experiment (LTEE) with *Escherichia coli* provides an optimal system for studying the nature of beneficial mutations and their pleiotropic effects for several reasons. Firstly, the founding *E. coli* strain has a rapid replication rate and well-characterized genetics and metabolism (Cooper 2002; Feist et al. 2007; Jeong et al. 2009). Secondly, effective techniques for short-term evolution, storage, and measuring fitness have already been established in this system (Lenski et al. 1991; Ostrowski et al. 2005; Gallet et al. 2012). Thirdly, the LTEE consists of a well-characterized evolutionary trajectory with rich genetic and phenotypic resources from previous studies, allowing for indirect manipulation of founding strains and a context for comparisons with previous studies. Lastly, of the few prior studies that have attempted to evaluate the direct and pleiotropic effects of individual beneficial mutations, several have been conducted in this system (Rozen et al. 2002; Ostrowski et al. 2005, 2008), allowing us to make direct comparisons between our findings and previous research.

Over the course of the first 50,000 generations of adaptation to glucose, the 12 replicate populations in the LTEE have increased their fitness to an average of more than 1.60 (Wiser et al. 2013). Several genetic and phenotypic targets of adaptation have been identified in this experiment, and a high degree of

molecular parallelism has been observed across replicate populations (Travisano et al. 1995b; Travisano and Lenski 1996; Woods et al. 2006; Ostrowski et al. 2008; Barrick et al. 2009). Although evolutionary tradeoffs have been a subject of investigation in the LTEE, these studies have yielded mixed results. While Biolog respiration assays initially suggested that growth on alternative carbon substrates had declined during the first 20,000 generations of adaptation to glucose (Cooper and Lenski 2000), a more recent study revealed that growth rates on many alternative carbon substrates actually increase during that time (Leiby and Marx 2014). Moreover, an analysis of these lineages at 50,000 generations suggests that the fitness trade-offs that were observed in the LTEE were primarily driven more by mutation accumulation in lines with elevated mutation rates, and not antagonistic pleiotropy (Cooper 2014; Leiby and Marx 2014).

The pleiotropic effects of individual beneficial mutations have been difficult to ascertain in the LTEE because they cooccur on evolved haplotypes. These mutations may produce distinct pleiotropic effects that vary in sign and may also interact with the growth environment (Flynn et al. 2013). To circumvent these problems, Ostrowski et al. performed short-term selection experiments to isolate a collection of the first beneficial mutants and study their pleiotropic effects. Most mutants were positively pleiotropic and the magnitude of pleiotropy correlated positively with the direct effects of the mutations in glucose (Ostrowski et al. 2005). However, some examples of antagonistic pleiotropy were also discovered, particularly in alternative environments whose methods of catabolism and uptake were most different from glucose, and these antagonistic effects did not correlate with the magnitude of the direct effect (Ostrowski et al. 2005). Sequencing of loci known to be under selection in this system revealed parallel mutations in many isolates, suggesting that selection had acted on only a few targets, but other loci may have also been targets of selection (Ostrowski et al. 2008).

While a few comprehensive studies have now been conducted to characterize the pleiotropic effects of adaptation, they suffer from one of two primary shortcomings: (a) they involve mostly large libraries of induced mutations (Remold and Lenski 2001; Bataillon et al. 2011; Hietpas et al. 2013), or (b) they involve the study of combinations of mutations (often unknown) in each genetic background (Travisano et al. 1995a,b; Cooper and Lenski 2000; Jasmin and Zeyl 2013; Leiby and Marx 2014). There remain few data on the direct and pleiotropic effects of individual, naturally arising beneficial mutations, especially where the genetic identity is also known.

Here, we used short-term selection experiments to isolate 21 mutations associated with improved fitness in a glucose-limited environment and characterized their fitness effects on five alternative carbon substrates. Our approach differs from previous work in three fundamental ways. First, we used a smaller

population bottleneck during daily transfers than the original LTEE and isolated mutations from both the winning and losing fraction of evolving populations. These modifications were intended to increase the variance in fitness among sampled beneficial mutations by increasing the probability of picking mutants of lesser benefit. Second, we isolated beneficial mutations from both the LTEE ancestor and an evolved clone with an approximately 1.30 fitness advantage relative to the ancestor. This design allowed us to study of the role of adaptive history on the effects of subsequent beneficial mutations. Lastly, we used whole-genome sequencing to identify the molecular basis of the beneficial mutations to associate specific mutations with their fitness effects. Overall, this project sought to broaden sampling of beneficial mutations, which are typically skewed toward few targets of large-effect (Gerrish and Lenski 1998; Orr 2003; Ostrowski et al. 2005, 2008; de Visser and Rozen 2006). We identified several novel molecular and phenotypic outcomes of adaptation to a simple glucose-limited laboratory environment, and present a more comprehensive perspective of the spectra of beneficial mutations and their pleiotropic effects.

Materials and Methods

BACTERIAL STRAINS AND CULTURE CONDITIONS

Two strains were used to initiate short-term selection experiments from naïve and glucose-adapted genotypes. The naïve genotype was the ancestral *E. coli* B ancestor used in the LTEE, REL606 (Lenski et al. 1991), which has been cured of all active plasmids and bacteriophage. The glucose-adapted genotype was isolated after 2000 generations of selection in the LTEE. This strain, named REL1206, has a fitness of ≈ 1.30 relative to *E. coli* REL606 as a result of at least five adaptive mutations (*rbsR*, *topA*, *spot*, *glmUS*, and *pykF*) (Lenski et al. 1991; Barrick et al. 2009; Khan et al. 2011; Flynn et al. 2013).

We used flow cytometry to rapidly detect subtle shifts in population structure due to an arising beneficial mutation in our selection experiments, requiring that we chromosomally mark each ancestral strain with both cyan (CFP) and yellow (YFP) fluorescent proteins, hereafter referred to as REL606-CFP, REL606-YFP, REL1206-CFP, and REL1206-YFP (SI Text). All selection experiments and competitions were carried out in Davis Minimal media supplemented with 25 mg/l of the appropriate carbon substrate (DM25) (Lenski et al. 1991; Travisano et al. 1995a; Ostrowski et al. 2005). Mutants recovered from freezer stock were preconditioned in LB for 24 h and then diluted 1:10,000 into DM25 for an additional 24 h of growth prior to initiating selection or competition experiments. In contrast to the LTEE, which was carried out using 50 ml glass flasks containing 10 ml of DM25 medium on a shaking incubator at 120 rpm, we conducted selections and competitions in 18 \times 150 mm glass capped tubes

containing 5 ml of DM25 medium maintained in a roller drum at 30 rpm. All serial transfers during experimental evolution were performed using 10,000-fold dilutions.

MUTANT COLLECTION AND ISOLATION

The rate and likelihood of capturing any given beneficial mutation in these short-term selection experiments depends on the beneficial mutation rate (μ_b) and the number of generations until the mutation reaches a sufficient frequency to increase the mean fitness of one marker population and cause it to deflect (Lenski et al. 1991). Both theory and experiments support the notion that reducing the bottleneck size will sample a broader set of beneficial mutations in isolation from other mutations and allow for the capture of more mutations of smaller effect that might otherwise be excluded by large-effect mutations (Gerrish and Lenski 1998; Wahl and Gerrish 2001; de Visser and Rozen 2005). Reducing the population bottleneck also has the effect of reducing the long-term genetic effective population size (N_E), defined as the harmonic mean of the population size during the growth cycle (Crow and Kimura 1970; Lenski et al. 1991). Thus, in addition to using a smaller culture volume during our short-term selection experiments, we reduced the transfer bottleneck by 100-fold from the LTEE conditions.

Selection experiments were conducted as follows. Following independent acclimation of each ancestral culture, 12 cocultures of oppositely marked REL606-CFP and REL606-YFP, and 12 cocultures of oppositely marked REL1206-CFP and REL1206-YFP were initiated via 10,000-fold dilution. As a result of the small fitness differences in the oppositely marked strains (SI Text), REL606 populations were initiated with a 3:1 ratio of CFP to YFP, and REL1206 populations were initiated with a 9:1 ratio of CFP to YFP. All populations were passaged by daily 10,000-fold dilutions into fresh DM25-glucose media, measuring the relative marker frequency every 3 days using a Millipore-8HT flow cytometer, until a marker divergence was observed or populations underwent 540 generations of selection (Fig. S2; Fig. S3).

This resulted in the collection of a total of 48 putative beneficial mutants (one winner and one loser from each population). The relative fitness of each of these 48 isolates was measured relative to the oppositely marked ancestor with twofold replication, and if increased fitness was observed, the isolate was kept for further analysis. These fitness assays identified a total of 24 putative beneficial mutations, including 16 derived from the REL606 background and eight derived from the REL1206 background.

DIRECT AND INDIRECT FITNESS ASSAYS

The relative fitness of all 24 mutants was measured in the following environments: DM25-glucose, DM25-N-acetyl-D-glucosamine (NAG), DM25-trehalose, DM25-galactose, DM25-melibiose, and DM25-maltose. We chose to vary carbon

substrates rather than other elements of the environment because of the wealth of knowledge about how these substrates are processed in *E. coli*, and evidence from previous studies showing that substrate transport is a central target of selection early in the LTEE (Travisano and Lenski 1996; Ostrowski et al. 2005). Resources were chosen to cover a range of potential mechanisms of outer and inner membrane transport. Glucose, NAG, galactose, and melibiose all use the OmpF porin for outer membrane transport, while trehalose and maltose use the LamB porin. For inner membrane transport, glucose, NAG, and trehalose all utilize the phosphotransferase system (PTS), while galactose, melibiose, and maltose use non-PTS pathways (Travisano and Lenski 1996; Ostrowski et al. 2005).

Fitness assays were carried out with each of the 24 beneficial mutants being assayed in all six alternative environments in four independent experiments (144 fitness assays per experiment, and $144 \times 4 = 576$ assays in total). Competitors were inoculated at a 1:1 ratio via a 100-fold dilution into fresh media and tracked by flow cytometry over 3 transfers (72 h). We used serial 100-fold dilutions rather than 10,000-fold dilutions to better discriminate mutations of smaller fitness effect. Fitness was calculated as described previously in this system using the ratio of the realized Malthusian parameters of the two competitors (Lenski et al. 1991; Ostrowski et al. 2005, 2008). In addition, raw cell counts at the beginning (N_i) and the end (N_f) of all competitions, along with relative growth rates (Δr), generations elapsed by the reference strain (G), and selection coefficients (s) for each competition are provided in Supplementary Dataset 1 to enable comparison with studies using different metrics of competitive fitness (Chevin 2011; Perfeito et al. 2014). Although the increased bottleneck size during our fitness assays affected the growth cycle of the populations, namely by decreasing the period of log-phase growth, the fact that all of the confirmed beneficial mutants in the selective environment were also beneficial in the these conditions suggests that the observed beneficial effects are mostly transitive.

DNA SEQUENCING AND ANALYSIS

Genomic DNA was extracted from each of the 24 putative beneficial mutations using the Wizard Genomic DNA Purification Kit (Promega Inc.). Following library preparation using a modified Illumina Nextera protocol (Baym et al. 2015), sequencing was performed to produce 151-bp, paired-end reads on an Illumina HiSeq2500 at the University of New Hampshire Hubbard Center for Genomic Studies. Raw reads were processed using Trimmomatic to remove Nextera PE adapter sequences and quality control was performed on all sequences with fastQC (Andrews 2010; Bolger et al. 2014). Processed reads were then analyzed using breseq to map them to the *E. coli* REL606 reference genome and identify mutations, using default settings (Deatherage and Barrick 2014).

An average of 97% of the reads from each isolate were mapped to the reference genome, resulting in an average coverage of $\approx 80 \times$ per isolate (Table S2). We first focused on high-confidence mutations for each isolate by extracting the raw calls from each isolate and comparing them to all other isolates with the same genetic background. If any mutation was called in more than 50% of the isolates derived from the same genetic background, it was considered a fixed progenitor mutation and was discarded. Next, we examined reports from Unassigned Coverage, New Junction Evidence, and Marginal Read Alignments for potential missed mutations in lower confidence regions or structural variants that are more difficult to identify. We also searched these lists for evidence of any of the genes that have previously been identified as common targets of selection in this system, including *rbs*, *spot*, *glmU*, *topA*, *pykF*, *nadR*, *pbp-radA*, and *hokB/sokB*, as well as mutations picked up in other lineages of the LTEE (Cooper et al. 2001, 2003; Woods et al. 2006; Ostrowski et al. 2008; Barrick et al. 2009). We also kept marginal calls for manual validation if at least 10 reads covered the site and there was more than 80% consensus for a novel nucleotide. Lastly, we manually validated all mutations using breseq's graphical output to verify our final set of mutations, a strategy that has previously been demonstrated to reduce the likelihood of false positives (Tenaillon et al. 2012). Manual validation led to us to discard the only two putative mutations that had been identified as marginal, resulting in a final mutation collection comprised only of high-confidence mutation calls. A summary of all mutations that were identified by breseq and how they were filtered is available in Supplementary Dataset 2.

To independently verify our mutation calls and identify any further mutations that were missed by breseq, we analyzed our sequencing data with a second pipeline designed for highly sensitive detection of base-substitution mutations, small insertion-deletion mutations, and large chromosomal changes (Lee et al. 2012; Sung et al. 2012a,b; Dillon et al. 2015). Briefly, this pipeline uses bwa and novoalign for alignment, then independently passages the alignments through the pattern-growth algorithm PINDEL, which identifies deletions, short-insertions, inversions, tandem duplications, large insertions, and unassigned breakpoints. Mutations are only called if they are independently identified by both bwa and novoalign (Lee et al. 2012; Sung et al. 2012a,b; 2015; Dillon et al. 2015).

STATISTICAL ANALYSIS

Following completion of all fitness assays and sequencing, only 21 (15 REL606 derived; six REL1206 derived) of the initial 24 mutants were used for the analyses presented below because the remaining three lacked any genetic or phenotypic evidence that they harbored a beneficial mutation. Specifically, the relative fitness of one REL606-derived genotype and two REL1206-derived genotypes was not significantly different from

1, independent of multiple comparisons, in any of the six environments tested (two-tailed *t*-test), and had no mutations identified in the whole-genome sequencing analysis pipeline described above.

All statistical analyses were performed in R Version 0.98.1091 using the Stats analysis package (R Development Core Team 2013). Independent two-tailed *t*-tests were used to test whether the average effect of each mutant in each environment differed significantly from 1 at a threshold *P*-value of 0.05. However, because there were 126 experimental conditions (21 mutants \times six resources), we applied a Benjamini–Hochberg correction to all of our *P*-values to ensure that our false-positive rate was below 5% (Benjamini and Hochberg 1995). Analyses of variance (ANOVAs) were performed on the direct effects of the beneficial mutations on glucose to test for effects of mutant fitness and block (date). We also performed ANOVAs on the entire array of pleiotropic effects of our beneficial mutations to test for effects of mutant fitness, resource, and mutant*resource interaction. Linear regressions were used to evaluate correlations between the direct effect of each mutation on glucose and their pleiotropic effects on each alternative resource. Shapiro–Wilkes tests were used to test for normality in the distribution of fitness effects for each environment. When the distribution was significantly non-normal, nonparametric Kruskal–Wallis tests were used to accompany ANOVAs, and nonparametric Spearman’s Rank correlations were conducted to accompany linear regressions.

Results

Experimental populations derived from the naïve REL606 genotype were evolved for up to 300 generations, at which point sufficient deviation from the marker-generated trajectory of all lineages was observed to warrant the inference that a beneficial mutation had arisen in at least one of the oppositely marked populations (Fig. S2). Given that these populations underwent daily bottlenecks of $\approx 25,000$ total cells, and the populations were founded at a ratio of 3CFP:1YFP, the maximum frequency at which a beneficial mutation could have arisen in a given background under these conditions is 0.00016. Using the formulas for the average selective rate constant ($r_{ij} = \bar{m}(S_{ij})$) the rate of change in the frequency of a beneficial allele per day ($\frac{dP}{dt} = r_{ij}P(1 - p)$), and the mean population-wide fitness ($\bar{W}(t) \cong 1 + \frac{r_{ij}P(t)}{\bar{m}}$) (Lenski et al. 1991), we can model the number of generations required for different values of S_{ij} that arise at a frequency of 0.00016 to increase marker wide fitness to a detectable level of 1.005. The minimum value of S_{ij} that can generate this marker wide fitness over 300 generations of evolution is the detection limit. For populations founded by the REL606 genotype, this value of S_{ij} is ≈ 0.04 . We note that this estimate assumes that all other cells within the winning and losing populations have not obtained any alternative beneficial mutations, and does not account for the initial waiting

time for a beneficial mutation to occur, so this value should be treated solely as a coarse estimate of our sensitivity to detect marker deflection. Moreover, not all mutants that we collected are subject to these detection limits as they may have been isolated from the losing marker fraction, or a portion of the winning fraction that was not driving the marker divergence (Table 1).

Deviation from the marker-generated trajectory took substantially longer in the glucose-adapted lineages founded from REL1206 (300–500 generations), and several populations did not diverge at all by ≈ 540 generations (Fig. S3). When sufficient deviation was observed, a mutant was isolated from both the winning and losing marker portions of the population. For the REL1206 lineages that did not diverge from the marker-predicted trajectory after ≈ 540 generations, the experiments were stopped and putative mutants were isolated from both marker portions, following the reasoning that clonal interference between beneficial mutations that had arisen in both backgrounds may have prevented divergence. Using the same logic described above, we estimate that the minimum S_{ij} required to detect marker deflection in the REL1206 populations was ≈ 0.02 , given the higher maximum frequency at which a beneficial mutation could arise (0.0004) and the longer time-frame of these evolution experiments (540 generations). However, even fewer of the mutants isolated from REL1206 are expected to be subject to these detection limits, as most were isolated prior to marker deflection or only shortly after (Table 1; Fig. S3).

In sum, a total of 24 putative beneficial mutants associated with adaptation to a minimal glucose environment were isolated by either marker deflection or preliminary fitness assays. Three of these putative beneficial mutants were subsequently discarded because of a lack of genotypic or phenotypic evidence that they harbored a beneficial mutation following whole-genome sequencing and fitness assays with higher replication, which left 21 confirmed beneficial mutants for the analyses presented below. Fifteen of these mutants were derived from REL606 (eight YFP, seven CFP) and six were derived from REL1206 (one YFP, five CFP). Whole-genome sequencing revealed only a single beneficial mutation in 15 of these mutants, while the genetic basis of adaptation was not identified for the remaining six beneficial mutants (Table 1; SI Text).

MAGNITUDE AND DISTRIBUTION OF DIRECT FITNESS EFFECTS

Beneficial mutants derived from the naïve REL606 genotype produced a mean relative fitness of 1.124, which is moderately higher than what has been observed previously for beneficial mutants derived from this genotype (Ostrowski et al. 2005), despite the fact that we attempted to isolate more mutations of smaller effect (see Materials and Methods). The average effect of mutations isolated from the winning background differed from those from

Table 1. Molecular basis and COG Cluster of all beneficial mutations isolated in this study from the naïve REL606 genotype and the glucose-adapted REL1206 genotype.

Ancestor	Isolate	Mutation type	Gene	Gene function	COG cluster
REL606	1	—	—	—	—
REL606	2	Missense (A43T)	<i>rho</i>	Transcription termination factor	Transcription
REL606	3	Missense (R609S)	<i>topA</i>	DNA topoisomerase I	Replication/repair
REL606	4	Missense (L183Q)	<i>ycbC</i>	Hypothetical	Unknown
REL606	5	Nonsense (E186*)	<i>prc</i>	Carboxy-terminal protease	Cell wall/membrane
REL606	6	Missense (M248R)	<i>tldD</i>	Protease	Unknown
REL606	7	Intergenic	<i>fabB/mnmC</i>	Synthase; methyltransferase	N/A
REL606	8	—	—	—	—
REL606	9	—	—	—	—
REL606	10	Missense (D99Y)	<i>ynfC</i>	Hypothetical	Unknown
REL606	11	Nonsense (E159*)	<i>yfgA</i>	Cytoskeletal protein RodZ	Cell wall/membrane
REL606	12	Missense (R334L)	<i>pykF</i>	Pyruvate kinase	Carb Metab./Trans.
REL606	13	Deletion (157)	<i>infB</i>	Translation initiation factor IF-2	Translation
REL606	14	Nonsense (E159*)	<i>yfgA</i>	Cytoskeletal protein RodZ	Cell wall/membrane
REL606	15	Deletion (491)	<i>prc</i>	Carboxy-terminal protease	Cell wall/membrane
REL1206	1	Synonymous (V198V)	<i>fdhE</i>	Formate dehydrogenase	Posttranslation Mod.
REL1206	2	—	—	—	—
REL1206	3	—	—	—	—
REL1206	4	Missense (H7Y)	<i>yfgA</i>	Cytoskeletal protein RodZ	Cell wall/membrane
REL1206	5	Deletion (301)	<i>cls</i>	Cardiolipin synthetase	Lipid metabolism
REL1206	6	—	—	—	—

the losing background, (winning: 1.143; losing: 1.071; Welch's two tailed *t*-test: $P = 0.032$). We also observed much greater fitness variance among mutants than had been observed previously (Ostrowski et al. 2005), with a range of 1.038–1.291 (Fig. 1). Mutant differences explained a significant fraction of the overall variance ($df = 14$, $MS = 0.0222$, $F = 80.86$, $P < 0.0001$), with the fitness of the average mutant differing by 7.4% from the mean. No significant effect of experimental block was observed ($df = 3$, $MS = 0.0002$, $F = 0.7380$, $P = 0.5350$). In addition, because a Shapiro–Wilkes test led us to question the normality of the distribution of fitness effects ($W = 0.8961$, $P = 0.0831$), we performed Kruskal–Wallis tests to further evaluate effects of mutant and block on fitness. These tests confirmed a highly significant mutant effect ($\chi^2 = 54.97$, $df = 14$, $P < 0.0001$) and demonstrated no significant block effect ($\chi^2 = 0.0833$, $df = 3$, $P = 0.9938$).

In contrast to the mutants from the naïve genotype, the mutants derived from glucose-adapted REL1206 ancestors produced a narrow range of fitness effects, with an average of 1.014 and a range of 1.000–1.033 (Fig. 1). Four of these beneficial mutants were isolated from a winning background and tended to be more fit (mean fitness = 1.021) than the remaining two that were isolated when no marker divergence was observed (mean fitness = 1.001; effect of isolation method, $P = 0.032$). Again, fitness among mutants varied significantly ($df = 5$, $MS = 0.0007$, $F =$

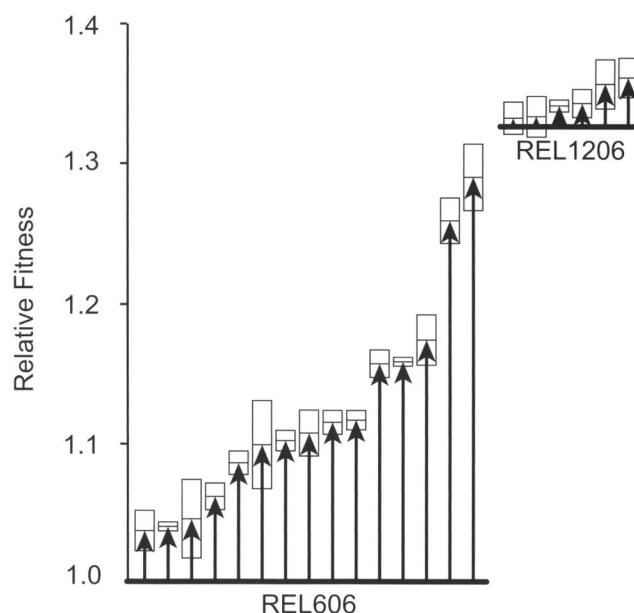


Figure 1. Magnitude of relative fitness increase in DM25-Glucose provided by each of the beneficial mutations isolated in this study. Each horizontal line represents the initial fitness of the founding genotype relative to the naïve REL606 ancestor across eight replicates, and each vector represents the mean fitness of each mutant relative to its founding genotype. Boxes represent 95% confidence intervals of the mean fitness across four replicates.

4.421, $P = 0.0113$), and no significant block effect was observed ($df = 3$, $MS = 0.0002$, $F = 1.374$, $P = 0.2889$). Nonparametric tests supported the same conclusions (Kruskal–Wallis tests; mutant effect: $\chi^2 = 12.98$, $df = 5$, $P = 0.0236$, block effect: $\chi^2 = 0.7733$, $df = 3$, $P = 0.8558$).

FORM AND DISTRIBUTION OF PLEIOTROPIC FITNESS EFFECTS

Pleiotropic effects of each mutant were assessed using fitness assays in five alternative carbon substrates alongside glucose. Pleiotropy was both common and predominantly positive among mutants of REL606, as the fitness of most mutants was also enhanced on alternative carbon substrates (Table 2; Fig. 2). Of the 15 mutants of REL606, four affected fitness in all five alternative environments, six affected fitness in four environments, four affected fitness in three environments, and one affected fitness in two environments (Table S1; Fig. S4). Thus, all beneficial mutations that favored during growth on glucose affected fitness in other resources (Table 2), and often in different ways depending on the mutation. Analysis of variance (ANOVA) revealed highly significant effects of mutant ($df = 14$, $MS = 0.1251$, $F = 277.7$, $P < 0.0001$), resource ($df = 15$, $MS = 0.1761$, $F = 390.9$, $P < 0.0001$), and mutant*resource interaction ($df = 70$, $MS = 0.0109$, $F = 24.09$, $P < 0.0001$).

Growth in resources with similar transport mechanisms to glucose tended to produce similar fitness levels (Table 2; Fig. 2) (Travisano and Lenski 1996; Ostrowski et al. 2005). As was the case in a previous study (Ostrowski et al. 2005), we found that when pleiotropy is positive, a significant correlation exists between the magnitude of the direct effect of a beneficial mutation and its pleiotropic effects in environments that are relatively similar to glucose (NAG, trehalose, galactose) (Table S3; Fig. S5). However, the direct fitness benefit of a mutation in glucose did not correlate with its fitness in melibiose or maltose, even when antagonistic mutations were eliminated from the analysis (Table S3; Fig. S5). Though these distributions of fitness effects in melibiose and maltose were significantly nonnormal (Shapiro–Wilkes test, Melibiose: $W = 0.8616$, $P = 0.0254$; Maltose: $W = 0.8632$, $P = 0.0269$), Spearman's rank correlations also demonstrated no significant relationship between the fitness in glucose and the fitness in melibiose or maltose (melibiose: $df = 14$, $P = 0.4039$; maltose: $df = 14$, $P = 0.1779$).

The subtle fitness benefits of the six REL1206 mutants in glucose correlated with small effects in other resources, although the difference in the magnitude of pleiotropic effects relative to REL606 was only statistically significant in NAG, galactose, and maltose (Welch's two tailed t -test; NAG: $P < 0.001$, trehalose: $P = 0.605$, galactose: $P = 0.004$, melibiose: $P = 0.143$, maltose: $P = 0.002$) (Fig. 2). Nonetheless, pleiotropy was still common and remained predominantly positive. No mutant significantly af-

fected fitness in all five alternative environments, but all mutants significantly affected fitness in at least one alternative environment (Table 2; Fig. S4). Despite the small sample size, ANOVA revealed significant effects of mutant ($df = 5$, $MS = 0.0089$, $F = 44.94$, $P < 0.0001$), resource ($df = 5$, $MS = 0.0067$, $F = 34.03$, $P < 0.0001$), and mutant*resource interactions ($df = 25$, $MS = 0.0060$, $F = 30.62$, $P < 0.0001$). However, fitness in glucose was only positively correlated with fitness in one other environment, NAG (Table S3; Fig. S6), and this general absence of correlated effects was further supported by nonparametric tests.

While most pleiotropic effects observed in this study were positive, we observed a few cases of severely antagonistic pleiotropy. Three REL606 mutations were deleterious in all alternative environments except NAG, with fitness ranging from 0.68–0.96 (Fig. 3B). Fitness in glucose for these mutants (1.05, 1.10, and 1.12) was comparable to others producing positive pleiotropy and thus not predictive of this antagonism (Figs. 1 and 2). One additional REL606 mutant was deleterious in maltose ($w = 0.92$). No mutants of REL1206 were consistently antagonistic, but two were deleterious in a single environment (Fig. 2; Table S1).

MOLECULAR BASIS OF BENEFICIAL MUTATIONS

As predicted by the phenotypic diversity in this study, the genetic basis of the beneficial mutations was also diverse. Importantly, we did not identify a single lineage that contained more than one fixed mutation, which supports the inference that all phenotypes trace to a single genetic change. However, we were unable to find the genetic basis of adaptation in three of the 15 REL606 derived beneficial mutants and three of the six REL1206 derived beneficial mutants (Table 1; Table S2). Our inability to find these mutations may be explained by the ~6% of the genome with insufficient coverage to call SNPs or because they involved structural changes that went undetected by breseq and pindel in low coverage regions. Although it is possible that we also missed secondary mutations in a lineage where a mutation was identified, this is unlikely to have occurred during the time frame of our experiments given the expected beneficial mutation rates and fixation times for beneficial mutations in this system (Lenski et al. 1991; Gerrish and Lenski 1998; Ostrowski et al. 2005).

Unlike previous studies of beneficial mutants, most causative mutations affected a unique gene. Even at the level of functional category, mutations occurred in genes belonging to eight different COG clusters, including Carbohydrate Metabolism and Transport, Cell Wall and Membrane, Lipid Metabolism, Post-Translational Modification, Replication and Repair, Transcription, Translation, and Unknown Function. Of these COG clusters, only the Cell Wall and Membrane cluster was hit more than once and it involved all examples of gene-level parallelism (Table 1). Namely, two presumed loss of function mutations in REL606 mutants disrupted the *prc* gene, which encodes a carboxy-terminal protease. Further,

Table 2. Summarized results of one-sample, two-tailed *t*-tests in each resource for REL606 derived (top) and REL1206 derived (bottom) mutants.

T-test result	Glucose	NAG	Trehalose	Galactose	Melibiose	Maltose
Significantly beneficial	14	14	9	11	4	8
Effectively neutral	1	1	4	1	8	3
Significantly deleterious	0	0	2	3	3	4
Significantly beneficial	2	3	4	2	1	2
Effectively neutral	4	3	1	4	4	4
Significantly deleterious	0	0	1	0	1	0

Mutants were designated as significantly beneficial or significantly deleterious based on a cutoff *P*-value of 0.05 following a Benjamini–Hochberg correction.

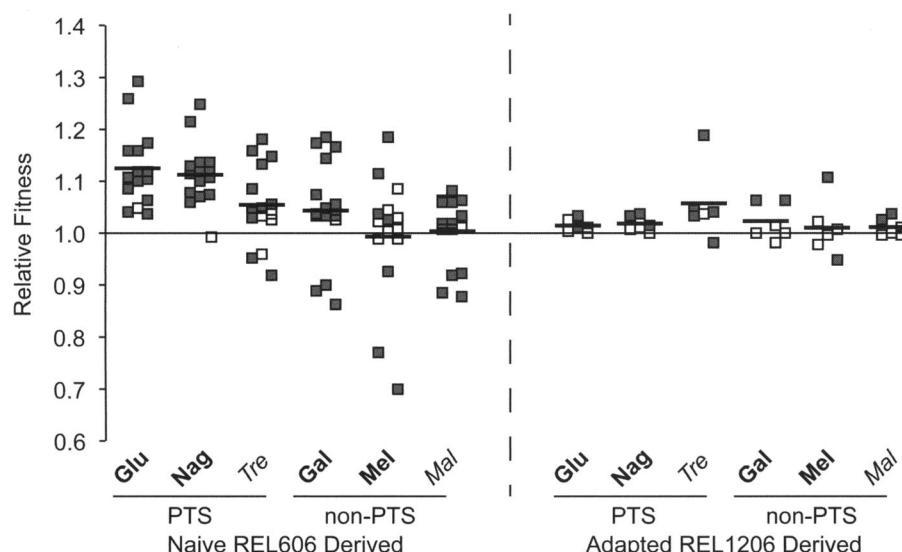


Figure 2. Relative fitness of each beneficial mutant against its founding genotype in DM25-Glucose (Glu) and five alternative environments: DM25-N-Acetyl-D-Glucosamine (NAG), DM25-Trehalose (Tre), DM25-Galactose (Gal), DM25-Melibiose (Mel), and DM25-Maltose (Mal). Each square represents the mean fitness of each mutant across four replicates, and each line represents the average fitness in each environment across all mutants ($n = 15$ for REL606; $n = 6$ for REL1206). Filled squares represent fitness values that are significantly different from 1, while open squares represent fitness values that are not significantly different from 1. Confidence intervals for each point are presented in Table S1. Resources are grouped by transport mechanism across the inner membrane (phosphotransferase (PTS) or nonphosphotransferase (non-PTS)) and outer membrane (OmpF (bold) or LamB (nonbold)). Each square represents the mean fitness of each mutant across four replicates, and each line represents the average fitness in each environment across all mutants ($n = 15$ for REL606; $n = 6$ for REL1206).

three beneficial mutations occurred in the *yfgA* gene encoding the cytoskeletal protein RodZ, two derived from REL606 and one derived from REL1206. All other mutations affected distinct genes, and only three of these genes (*pykF*, *topA*, and *infB*) have previously been identified as molecular targets of adaptation in the LTEE (Cooper et al. 2001, 2003; Woods et al. 2006; Ostrowski et al. 2008; Barrick et al. 2009; Khan et al. 2011).

MOLECULAR AND PHENOTYPIC PARALLELISM

Despite the overall lack of parallelism observed here, the two examples were especially interesting because the *prc* mutants of REL606 were most deleterious in alternative resources and the *yfgA* mutants of REL606 were most beneficial in glucose.

Further, a third, distinct *yfgA* mutant was isolated from REL1206 and produced dramatically different direct and pleiotropic effects than the two REL606-derived *yfgA* mutations (Fig. 3).

The two *prc* mutants of REL606 included a nonsense mutation (E186*) and a 1-bp deletion affecting the 491st amino acid. Both of these mutations are expected to truncate the protein, but their fitness phenotypes are distinct, presumably because the more complete product produced by the second mutant retains partial function (Fig. 3A). Interestingly, the deletion mutant is significantly more beneficial in glucose and reduces the pleiotropic costs of adaptation (glucose: $t = 4.442$, $df = 3$, $P = 0.0212$; NAG: $t = 4.810$, $df = 3$, $P = 0.0171$, and melibiose: $t = 11.97$, $df = 3$, $P = 0.0013$). In contrast, the two *yfgA* mutants of REL606 involved

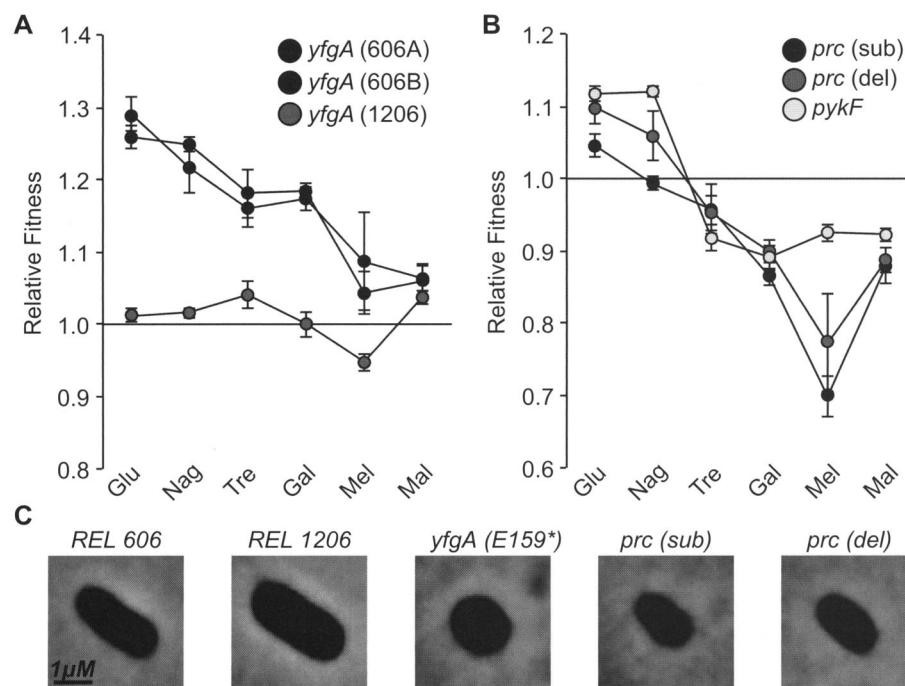


Figure 3. Relative fitness of parallel high benefit (A) and highly antagonistic (B) beneficial mutants in DM25-Glucose (Glu), DM25-N-Acetyl-D-Glucosamine (Nag), DM25-Trehalose (Tre), DM25-Galactose (Gal), DM25-Melibiose (Mel), and DM25-Maltose (Mal). Each point represents the average fitness across four replicates and error bars represent 95% confidence intervals of those measurements. (C) Phase contrast images of both ancestral clones and the mutants affecting cell wall biosynthesis. All images are magnified to scale.

genetically identical nonsense mutations (E159*). As expected, these mutants were phenotypically indistinguishable in all of the environments assayed and were positively pleiotropic (Fig. 3B). This *yfgA* mutation demonstrates that large gains in fitness can sometimes be achieved with few pleiotropic costs (Fig. 2). The third *yfgA* mutation (H7Y) that occurred in the REL1206 background was a distinct missense mutation rather than nonsense. Interestingly, both *prc* and *yfgA* mutations produced smaller cells (Fig. 3C) despite the dramatic differences in their fitness effects.

Lastly, three REL606 mutants acquired mutations in *pykF*, *topA*, and *infB*, genes that have been identified as targets of selection in the LTEE. The nonsense mutation in the *pykF* gene (R334L) was the third most antagonistically pleiotropic mutant and reduced fitness in all alternative environments except NAG (Fig. 3B). The missense mutation in *topA* (R609S) was generally positively pleiotropic as it increased fitness in all environments except melibiose (Table S1). Lastly, the 1-bp deletion in the *infB* allele was highly beneficial in glucose (1.17), positively pleiotropic in NAG, trehalose, and galactose, but was neutral in melibiose and deleterious in maltose (Table S1).

Discussion

Despite their importance in countless biological processes, studies addressing the nature and pleiotropic effects of beneficial mutations remain limited (Elena and Lenski 2003; Orr 2003; Kassen

and Bataillon 2006; Eyre-Walker and Keightley 2007; Bataillon et al. 2011; Good et al. 2012; Rice et al. 2015). Here, we built upon established methods for the detection and analysis of beneficial mutations using current technologies for cell counting and sequencing. Our approach enabled a diverse collection of beneficial mutants that includes some of the variation that may be typically lost due to random sampling and clonal interference in large populations (Gerrish and Lenski 1998; Ostrowski et al. 2005; Levy et al. 2015). We determined the direct and pleiotropic fitness effects of these mutants in six environments and identified the genetic basis of adaptation using whole-genome sequencing.

In an attempt to broaden sampling of beneficial mutations, we decreased the bottleneck size of our short-term selection experiments, isolated beneficial mutations from both a naïve and glucose-adapted ancestor, and screened cells from both the winning and losing populations to produce our final collection of 21 single-step beneficial mutations (Gerrish and Lenski 1998; Wahl and Gerrish 2001; de Visser and Rozen 2005). As expected, this resulted in diverse phenotypic responses to selection in DM25-glucose, with an average direct fitness effect of 1.124 for mutants derived from the naïve ancestor (REL606) and an average direct fitness effect of 1.014 for mutants derived from the glucose-adapted ancestor (REL1206). Individual REL606 mutants differed on average from this fitness mean by 7.4%, while REL1206 mutants differed from the overall mean by 1.2%. In contrast, a previous collection of 27 beneficial mutants collected from

REL606 in larger populations diluted only 100-fold each day exhibited a fitness deviation of only 0.026%, despite having a similar overall mean fitness to our collection (1.096) (Ostrowski et al. 2005). We are unaware of a comparable dataset for our REL1206 mutants, but theory and experiments support the decreased magnitude of fitness benefits of mutations in adapted genotypes owing to diminishing returns epistasis (Chou et al. 2011; Khan et al. 2011; Rokyta et al. 2011; Good et al. 2012; Kryazhimskiy et al. 2014; Nahum et al. 2015). It is worth noting that two of the four beneficial REL1206 mutants isolated via marker deflection had selection coefficients below what we would expect to detect with our methods. It is therefore conceivable that frequency-dependent selection allowed for their more rapid invasion and early impact on the mean population fitness of their respective marker population. Alternatively, it is possible that these mutants were aided by the presence of other beneficial mutations in different cells of the same marker population, or that these mutants themselves did not represent the most beneficial mutant in their marker population.

Although the predominant form of pleiotropy observed in this study was positive, not all beneficial mutants were positively pleiotropic. Most mutants exhibited universally positive pleiotropy in the five alternative environments, but others exhibited broadly antagonistic effects. While many evolutionary models have assumed that pleiotropy is mostly antagonistic (Fisher 1930; Lande 1983; Orr and Coyne 1992; Otto 2004), positive pleiotropy seems to be the rule rather than the exception when beneficial mutants are assayed on alternative carbon substrates (Ostrowski et al. 2005; Lee et al. 2009). Prior studies have also identified that adaptation can be resource-specific and can influence the magnitude and direction of pleiotropy in this system. For example, resources that share similar mechanisms of inner and outer membrane transport with glucose have been shown to permit similar fitness levels, while resources with dissimilar transport mechanisms reveal neutral or antagonistic effects (Travisano et al. 1995b; Travisano and Lenski 1996; Ostrowski et al. 2005). Adaptation appears to be somewhat related to transport among the REL606 mutations studied here, but this does not appear to be the case for REL1206 mutants, which have a similar distribution of pleiotropic effects in all alternative carbon substrates regardless of their transport mechanism (Fig. 2). Moreover, some REL1206 mutants were more fit in foreign environments than in glucose, despite little similarity in the resource transport mechanism (Table S1). This observation may indicate that the targets of adaptation have changed over the course of 2000 generations of prior adaptation to glucose. Furthermore, while antagonistic pleiotropy was relatively rare (Ostrowski et al. 2005; Lee et al. 2009), it was almost exclusively limited to three particular mutants (Fig. 3B), suggesting that different paths of adaptation yielding similar fitness benefits in the selective environment may produce vastly different evolutionary trade-offs (Rodriguez-Verdugo et al. 2014).

The genetic basis of the beneficial mutations collected in this study was also diverse and does not support the oligogenic model of adaptation discussed in previous studies (Wood et al. 2005; Ostrowski et al. 2008). Among 15 beneficial mutants tracing to a single, known mutation, only two cases of parallelism at the gene level were observed (Table 1). Moreover, we isolated mutations in only three genes that were previously identified as targets of adaptation in the LTEE (*pykF*, *topA*, and *infB*), despite the analysis of several thousand generations of whole-genome sequencing data from this project (Cooper et al. 2001, 2003; Woods et al. 2006; Ostrowski et al. 2008; Barrick et al. 2009; Khan et al. 2011). The remaining 12 mutants represent novel selective targets for adaptation in an environment that closely resembles that of the LTEE. These novel mutations occurred in genes belonging to six different COG clusters, and include missense, nonsense, synonymous, and indel mutations (Table 1). Collectively, this study suggests that that the number of mutations producing detectable benefit under this selective regime may be greater than previously expected, and this increase may be a product of sampling from populations evolved with a stronger bottleneck. In addition, subtle differences in our model such as the reduced culture volume, longer exponential phase following a more severe bottleneck, and altered oxygenation from growing in test tubes may explain the novel mutational targets. It seems likely that the five mutants with relative fitness improvements greater than 1.10 (with mean fitness ranging from 1.14 to 1.29) could represent new targets of selection in our model as compared to the LTEE, in which single mutations producing benefits greater than 1.10 were rarely observed (Lenski et al. 1991; Lenski and Travisano 1994; Barrick et al. 2009; Wiser et al. 2013).

A few key generalizations can be made from these adaptive genotypes and their fitness effects. First, the direct effects of beneficial mutations derived from naïve genotypes on glucose are substantially higher than those derived from glucose-adapted genotypes (Welch's two tailed *t*-test; $P < 0.001$) (Fig. 1). Second, the pleiotropic effects of beneficial mutations on alternative carbon substrates are generally positive from both ancestors, but these pleiotropic effects were typically greater for mutants of naïve genotypes (Welch's two tailed *t*-test; NAG: $P < 0.001$, trehalose: $P = 0.605$, galactose: $P = 0.004$, melibiose: $P = 0.143$, maltose: $P = 0.002$) (Fig. 2). Lastly, mutations in a variety of genes can be highly adaptive in this selective regime, but fewer so in adapted genotypes (Table 1; Fig. S2; Fig. S3). How these findings relate to Fisher's geometric model of adaptation is an important, yet contradictory perspective. On the one hand, these results are consistent with the model in that the selected effects of beneficial mutations decline as fitness of the population increases and that pleiotropy is universal (Orr 2006; Blanquart et al. 2014; Matuszewski et al. 2014). On the other hand, and arguably more significant, the sign of the pleiotropic effects in this study

disagrees with many tradeoff models predicted by the geometric model, in which pleiotropy is expected to be mostly antagonistic (Orr 2000; Otto 2004; Lourenço et al. 2011).

Exceptions to these generalizations are equally important as they can have dramatic consequences on evolving populations. The two genes in which more than one beneficial mutation was detected produced contradictory effects. In one case, mutants of *yfgA* produced large direct benefits without any pleiotropic costs, and in the other case, mutants of *prc* produced highly antagonistic pleiotropy with only moderate direct benefits. Three different mutants affected *yfgA*, which encodes the cytoskeletal protein RodZ, a key component of cell wall biosynthesis. Both *yfgA* mutations in REL606 were identical nonsense mutations that were the most beneficial (1.29 and 1.26) of all mutants isolated in this study and displayed positive or neutral pleiotropic effects in all alternative environments (Fig. 3A). A third *yfgA* missense mutation of REL1206 produced a fitness gain of only 1.012 with limited pleiotropic effects (Fig. 3A). Individual mutations producing a fitness gain of greater than 1.25 are expected to be extremely rare, as illustrated by the fact that single *yfgA* mutants in REL606 nearly reached the relative fitness of REL1206, which harbors at least five beneficial mutations acquired over the course of 2000 generations of selection in glucose (Fig. 1; SI Text). This result implies that single beneficial mutations can occasionally produce large fitness gains with limited pleiotropic costs. The lesser advantage of the *yfgA* mutant of REL1206 in all environments could be explained either by its less severe missense mutation or by diminishing returns epistasis (Chou et al. 2011; Khan et al. 2011; Rokyta et al. 2011; Good et al. 2012; Kryazhimskiy et al. 2014; Nahum et al. 2015). However, it is also noteworthy that this *yfgA* missense mutation was deleterious in melibiose (Fig. 3; Table S1), suggesting that both the magnitude and direction of pleiotropy can vary as a result of genetic background (Flynn et al. 2013). The two *prc* mutations are predicted to abolish the function of a periplasmic carboxyl terminal protease (Table 1). Along with a missense mutation in *pykF* (R334L), which encodes a pyruvate kinase, these three mutants account for 11 of the 12 cases of significantly antagonistic pleiotropy among REL606 mutants. Each mutant was deleterious in all three non-PTS resources, with relative fitness ranging from 0.70 to 0.92, and the *prc* nonsense and *pykF* mutants were also significantly deleterious in trehalose (Fig. 3B; Table S1).

Despite the opposing pleiotropic effects of mutations in *yfgA* and *prc*, both genes are involved in cell wall biosynthesis and produced similar impacts on cell morphology (Fig. 3C). The *yfgA* gene encodes a cytoskeletal protein that helps to maintain the rigid rod morphology of *E. coli* cells by anchoring to the cytoplasmic membrane and interacting with penicillin binding proteins (PBPs) to help synthesize the peptidoglycan layer of the cell wall (Jeong et al. 2009; Philippe et al. 2009). The *yfgA*

product interacts with MreB to form the actin cytoskeleton and constrain peptidoglycan synthesis to the periplasm (Shiomi et al. 2008; van den Ent et al. 2010). Interestingly, *mreB* mutations can confer a similar fitness benefit via cell size changes in *E. coli* (Monds et al. 2014). The *prc* carboxyl terminal protease activates PBPs to synthesize the peptidoglycan layer (Hara et al. 1991; Tadokoro et al. 2004). Loss of function mutations in *yfgA*, *mreB*, and *prc* would therefore be predicted to synthesize a more limited peptidoglycan layer and thus produce more spherical cells, which we indeed observed (Fig. 3C). These results add to numerous reports of beneficial changes in cell morphology in the LTEE, where loss in rod-shape morphology and cell size increases appear to scale linearly with fitness (Philippe et al. 2009; Monds et al. 2014). Although these changes in cell morphology are expected to be highly pleiotropic by affecting nutrient uptake, cell survival, and growth, mutations in the *prc* gene may be more predisposed to antagonistic pleiotropy because of this gene's involvement in the activation of other proteins along with PBPs (Hara et al. 1991; Tadokoro et al. 2004). In sum, although the amount of genetic parallelism observed in this study was limited, the few examples we did observe suggest that strong selection on a particular target may nevertheless produce divergent evolution in niche breadth, with some mutations resulting in pleiotropic gains in fitness, and others resulting in pleiotropic loses.

Despite growing evidence of the universality and diversity of pleiotropy, which is the root cause of tradeoffs produced by selection, we still know relatively little about the commonality and the form of pleiotropic effects of beneficial mutations, even in well-characterized systems (Cooper and Lenski 2000; Cooper et al. 2001; Dudley et al. 2005; Ostrowski et al. 2005; Kassen and Bataillon 2006). We have shown that a diverse collection of beneficial mutations can generate highly divergent pleiotropic outcomes. On average, the magnitude of pleiotropic effects scaled with the magnitude of the fitness increase in the selected environment, and hence mutations affecting the less adapted ancestor were more pleiotropic, and mutations affecting the more adapted ancestor were less pleiotropic. Nevertheless, some mutants that were beneficial on glucose were highly antagonistic on other carbon sources. Ultimately, these findings suggest that a variety of beneficial mutants are accessible at different points on the fitness landscape, and the success of a particular mutation can either expand the fundamental niche through positive pleiotropy, or lead to specialization via loss of fitness on alternative carbon substrates. However, while our mutants were collected in a simple environment with a single carbon source and constant temperature, nature surely presents greater environmental heterogeneity. Thus, which of these evolutionary paths is followed, and the eventual shape of the niche, may depend on the influence of environmental fluctuations that expose previously hidden pleiotropic effects that favor or disfavor contending mutations.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.f7p18.

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Supporting Information

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Table S1. Relative fitness, 95% confidence intervals, and two-tailed t-test statistics used to test for each of the twenty-one mutants in each resource studied, following Benjamini-Hochberg correction (BH).

Table S2. Sequencing statistics and mutations identified for each of the 24 putative beneficial mutations.

Table S3. Linear regression statistics of the relationship between direct fitness in DM25-glucose and pleiotropic fitness in five alternative resources.

Figure S1. Relative fitness difference between naïve REL606 and glucose-adapted REL1206 in the selective environment of the LTEE (10 ml flasks) and the selective environment employed in this study (5 ml tubes).

Figure S2. Evolutionary trajectories of each of the twelve REL606 short-term evolutions inoculated at a ratio of 3 CFP : 1 YFP.

Figure S3. Evolutionary trajectories of each of the twelve REL1206 short-term evolutions inoculated at a ratio of 9 CFP : 1 YFP.

Figure S4. Histogram demonstrating the number of alternative environments in which each beneficial mutation significantly affected fitness ($w \neq 1$) based on two-tailed t-tests with a Benjamini-Hochberg correction for REL606 (A) and REL1206 (B).

Figure S5. Linear regressions of the relationship between direct fitness in DM25-glucose and pleiotropic fitness in five alternative resources for the sixteen beneficial mutations isolated from REL606 (see Table S3 for statistics).

Figure S6. Linear regressions of the relationship between direct fitness in DM25-glucose and pleiotropic fitness in five alternative resources for the six beneficial mutations isolated from REL1206 (see Table S3 for statistics).