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Adaptation of *Escherichia coli* to glucose promotes evolvability in lactose

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The selective history of a population can influence its subsequent evolution, an effect known as historical contingency. We previously observed that five of six replicate populations that were evolved in a glucose-limited environment for 2000 generations, then switched to lactose for 1000 generations, had higher fitness increases in lactose than populations started directly from the ancestor. To test if selection in glucose systematically increased lactose evolvability, we started 12 replay populations—six from a population subsample and six from a single randomly selected clone—from each of the six glucose-evolved founder populations. These replay populations and 18 ancestral populations were evolved for 1000 generations in a lactose-limited environment. We found that replay populations were initially slightly less fit in lactose than the ancestor, but were more evolvable, in that they increased in fitness at a faster rate and to higher levels. This result indicates that evolution in the glucose environment resulted in genetic changes that increased the potential of genotypes to adapt to lactose. Genome sequencing identified four genes—*iclR*, *nadR*, *spoT*, and *rbs*—that were mutated in most glucose-evolved clones and are candidates for mediating increased evolvability. Our results demonstrate that short-term selective costs during selection in one environment can lead to changes in evolvability that confer longer term benefits.

KEY WORDS: **Adaptation, evolvability, experimental evolution.**

Historical contingency—when the evolutionary history of a population affects its subsequent evolution—can profoundly influence evolutionary outcomes (Gould 1989). For example, populations with different evolutionary histories might respond differently when they are selected in a common environment due to epistatic interactions that cause new mutations to have effects that depend on different genetic backgrounds (Barrick et al. 2010, Perfeito et al. 2013; Harms and Thornton 2014; Kryazhimskiy et al. 2014). To the extent that historical contingency is influential, it can mean that predicting evolutionary outcomes will require some knowledge of the past selection of a population.

In practice, it is difficult to distinguish between the action of contingent and chance effects in shaping evolutionary outcomes. To do so requires a comparison of the replicated evolutionary response of populations with known histories evolving in identical environments. Microbial evolution experiments represent one way to meet these conditions. Indeed, several such studies have found evidence for contingency. For example, replicate populations that diverged from one another during adaptation to glucose responded differently, largely erasing those differences, following subsequent selection in maltose (Travisano et al. 1995). A similar result was seen in a recent study that identified the current fitness of a population as the primary driver of subsequent evolutionary potential (Kryazhimskiy et al. 2014). Other examples include finding differences in the evolvability—usually measured as the extent of fitness improvement over a defined time interval—of bacterial and viral populations that were initially isogenic except for different deleterious or beneficial mutations (Burch and Chao 2000; Moore et al. 2000; Cuevas et al. 2009; Barrick et al. 2010; Salverda et al. 2011; Woods et al. 2011; Perfeito et al. 2013; Plucain et al. 2014) or degree of specialization (Buckling et al. 2003), and cases where early "potentiating" mutational events are required for the evolution of subsequent phenotypic novelty (Blount et al. 2012; Meyer et al. 2012).

The studies cited above have clearly identified a role of history in influencing future evolutionary potential. In most cases, however, this history is either the result of: chance differences in mutation order leading to differences between genotypes despite selection in a common environment (Travisano et al. 1995; Blount et al. 2008; Salverda et al. 2011; Woods et al. 2011; Kryazhimskiy

et al. 2014) or deliberately selected or engineered differences in genotypes (Moore et al. 2000; Barrick et al. 2010; Perfeito et al. 2013). Alternatively, genotypes with different selective histories have been compared, but without replication at the level of the past selective environment (Cuevas et al. 2009). It has rarely been possible to test for a systematic effect of past selection environments on future evolvability (Buckling et al. 2003, McBride et al. 2008; Bedhomme et al. 2013).

We previously presented an observation consistent with adaptation of *Escherichia coli* to a glucose-limited environment increasing the rate of subsequent adaptation to an otherwise identical lactose-limited environment (Satterwhite and Cooper 2015). Here we carry out an experiment that repeats and extends the basis of this observation. We find that glucose-evolved populations had a consistent advantage in subsequent adaptation to lactose, increasing fitness at a higher rate and to a higher final level, relative to populations started from the original ancestral strain. This result held even when populations were started from individual glucose-evolved clones, indicating that the advantage was a property of individual genotypes. We interpret these results as evidence that adaptation to one or more components of the glucose environment resulted in a repeatable increase in evolvability in the lactose environment.

Materials and Methods

BACTERIAL STRAINS AND SELECTION EXPERIMENTS

The initial evolution experiment consisted of six *E. coli* populations evolved in each of seven selection treatments (Cooper and Lenski 2010). These treatments differed only in the nature of the limiting carbon source(s) added to Davis Mingioli (DM) medium. All populations were founded by strain REL606 or strain REL607, an otherwise isogenic derivative of REL606 that is able to grow on arabinose (Ara+) (Lenski et al. 1991). Here, six populations—referred to as founder populations—previously evolved for 2000 generations in glucose (175 μg/ml) supplemented medium were used to found a series of new replay populations that were selected in lactose (210 μg/ml) supplemented medium. These replay populations comprised (1) replication of each of the six founder populations into six new populations (36 total populations), (2) isolation of a randomly chosen clone from each founder population that was used to found six new populations (36 total populations). In addition, 18 control populations were founded from the ancestral strains REL606 or REL607. A schematic of this experimental design is presented in Figure 1. A derivative of REL606 that encodes green fluorescent protein (GFP) was used as a reference strain in competition experiments (Zhang et al. 2012).

A new evolution experiment involving the 90 populations described above was carried out using the same protocols and growth conditions used in the original evolution experiment (Cooper and Lenski 2010). Briefly, populations were grown in 1 ml DM supplemented with 210 μg/ml lactose (DM210 lactose) for 1000 generations by daily transfer of 10 μl samples. Propagation was in 96-deep well polypropylene plates. As far as possible, populations with different Ara marker states were arranged in a checker board pattern to facilitate checking for cross-contamination of wells. Every 13 days cultures were stored at −80°C in 20% glycerol, and tested for cross-contamination by inoculating 10 μl from each well onto DM + arabinose agar and plating onto tetrazolium arabinose (TA) indicator medium.

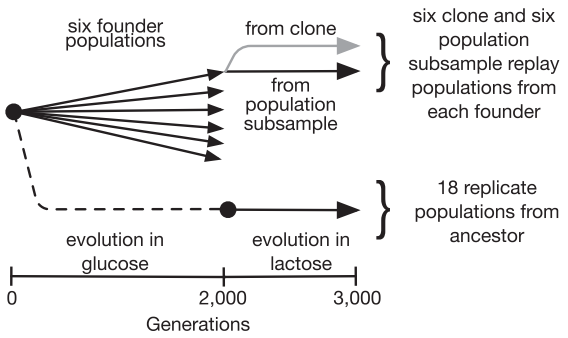

Figure 1. Schematic of experimental design. Six replicate populations were started from an ancestral strain and evolved for 2000 generations in a glucose-limited environment. Each of these founder populations was used to found 12 new populations, six started from a random clone and six from a population subsample. These new populations and 18 populations started directly from the ancestor were evolved in lactose for 1000 generations and their fitness compared.

FITNESS COMPETITIONS

Relative fitness of evolved populations was measured at 0, 500, and 1000 generations of evolution using competitive growth assays in the DM210 lactose selection environment. All assays were carried out as complete experimental blocks by using three 96-well deep well plates, one for each assayed evolutionary time point. Prior to each competition, experimental and reference fluorescent ancestor (one per competition) populations were acclimated to the selection environment over two daily transfer cycles. On the third day, paired reference and experimental populations were combined at 1:1 and a 1:100 dilution of this mix was used to start competition cultures. An Accuri C6 (Becton Dickinson, NJ) flow cytometer was used to measure the ratio of competing strains. To do this, competitions were diluted 1:100 from the original 1:1 mix (t0) or from the end of one day of competition (t1) into a 96-well polystyrene plate where each well contained a solution of 150 nM SYTO17 fluorescent dye and 10% DM made without MgSO4 or thiamine. Cells were separated from background noise on the basis of SYTO17 fluorescence, which indicates the presence of nucleic acid. Reference cells were distinguished from

their competitors on the basis of their GFP fluorescence signal. At least 5000 events were collected from each sample time point. The percentage of each competitor at the beginning and end of each competition was used to estimate fitness as the ratio of Malthusian parameters (Satterwhite and Cooper 2015). Using the percentage, rather than density, of competitors to calculate this ratio will underestimate fitness differences if evolved populations consistently reach lower stationary phase densities than ancestral populations. Although we cannot exclude this possibility, we were not able to find evidence for it in a previous study examining these populations (Satterwhite and Cooper 2015).

MUTATION RATE ESTIMATES

Mutation rate to rifampicin resistance was measured for the ancestor and each of the six clones isolated from the founder glucose-evolved populations. To do this, freezer stocks were grown overnight at 37°C in lysogeny broth (LB) and then diluted to give an inoculum of approximately $\sim 10^3$ cells that was added to each of 23 fresh LB cultures. After overnight growth, a sample from each replicate population was plated onto LB + rifampicin (100 μg/mL) to determine the number of Rif^r mutants. Samples from three populations were also plated onto LB to determine total cell density. Colonies were scored after 24 hr incubation at 37°C. Analysis was carried out using the Ma-Sandri-Sarkar Maximum Likelihood Estimator method implemented in the FALCOR online calculator (http://www.keshavsingh.org/protocols/FALCOR.html) (Hall et al. 2009).

GENOME SEQUENCING

Illumina library preparation was carried out using the NexteraXT kit protocol, except that reaction volumes were reduced by a factor of four, or the TruSeq kit protocol. Sequencing was performed on HiSeq2000 and NextSeq500 machines. Mutational changes occurring in evolved clones were identified by comparison to the previously sequenced ancestral strain, REL606, using BRESEQ (Barrick et al. 2009). A mutation in *recD* (V10A) was found in the three clones derived from REL607 and was subsequently identified to be present in our stock culture of that ancestral strain. Extensive competition experiments between the Ara- REL606 and our Ara+ derivative indicate that the mutation is neutral, so we omit it from further consideration (data not shown).

STATISTICAL ANALYSES

Statistical analyses were performed in R version 3.1.1 (R Core Team 2013). Differences in evolvability were determined by using the package NLME4 to fit a linear mixed effects model (Bates et al. 2014). Replicate populations were nested within their initial founder population or genotype as appropriate and were fitted with random intercepts. Models were fit with and without the effect of interest, usually whether populations were evolved directly from the ancestor or from glucose-evolved founder genotypes, and their explanatory power compared using a χ^2 test with one degree of freedom. Replay populations started from the glucose-evolved founders started off at a lower fitness in lactose than did populations started from the ancestor, which can have a systematic effect on evolvability (Barrick et al. 2010; Kryazhimskiy et al. 2014). It was not possible to account for this effect by using initial fitness as a covariate because it is completely confounded with population history. For this reason, in most cases we focus on comparing the final fitness of replay populations, rather than overall change in fitness, because a higher final fitness of initially less fit populations clearly indicates a meaningful difference in evolvability due to genotype. Initial fitness was included as a covariate in analyses that test for differences between founder populations or clones.

Results

We previously reported the evolution of six replicate populations that were selected in a glucose-limited environment for 2000 generations and then transferred to selection in an otherwise identical lactose-limited environment (Satterwhite and Cooper 2015). Here, we follow up the observation that the six glucose-evolved populations improved in fitness more rapidly when switched to selection in lactose than did populations started directly from the same ancestral strain. Following transfer to lactose selection, glucose-evolved populations increased in fitness measured in lactose by an average of 32.1% after 1000 generations compared to 23.1% for replicate populations derived directly from the ancestor. All fitness measurements are relative to the same ancestral strain. This observation is consistent with a period of selection in glucose systematically increasing the potential to adapt—which we here refer to as an increase in evolvability—to the lactose environment (ANOVA $F_{1,74} = 10.95$, $P = 0.001$). Increased evolvability may be due to mutations that commonly accumulate during selection in glucose acting to increase subsequent evolvability in lactose. Alternatively, higher evolvability might reflect a higher initial genetic variance in fitness within glucose-evolved populations relative to populations started from the ancestor. Higher variance in fitness is expected to increase the rate of adaptive response independently of any change in the adaptive potential of a specific genotype (Fisher 1999).

To disentangle the effect of genotype specific evolvability from the effect of differences in initial population-level genetic variation on subsequent adaptation to lactose, we repeated the part of our previous evolution experiment involving adaptation to lactose including replication at the level of each of the six founder glucose-evolved populations. From each founder population 12 new "replay" populations were started, six from population subsamples and six from a single randomly selected clone. These replay populations and 18 reference populations started directly

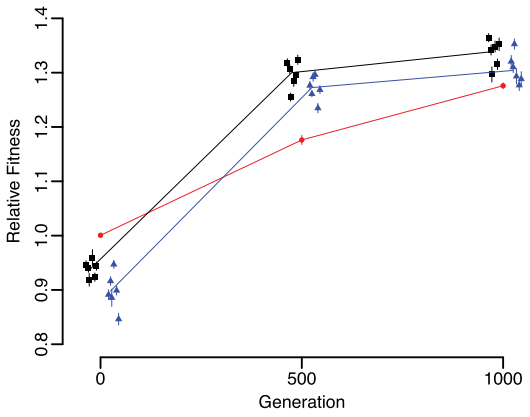

Figure 2. **Fitness change in the lactose-limited environment of populations evolved in this study. Each symbol represents the mean of six replicate populations started from the same founding clone or population subsample (blue and black, respectively) or of 18 replicate populations started directly from the ancestor (red). For each treatment, solid lines connect the grand mean across assayed time points. Error bars indicate SEM. All fitness measurements are made relative to the ancestor. Points are offset slightly on the x-axis to increase legibility.**

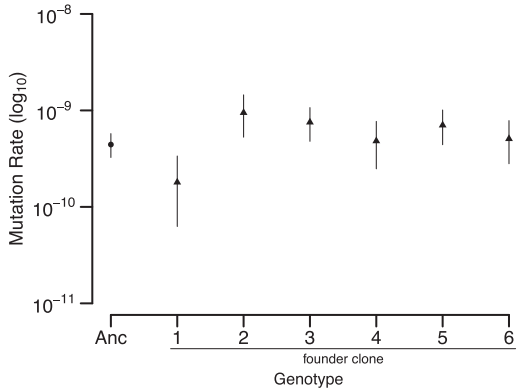

Figure 3. **Mutation rate to rifampicin resistance of the ancestor and the six glucose-evolved founder clones. Symbols and error bars represent the estimated mutation rate to rifampicin resistance and 95% confidence intervals.**

from the original ancestral genotype were evolved for 1000 generations in lactose.

After 500 generations of evolution in lactose, the fitness of the replay populations started from both clones and population subsamples were significantly greater than that of populations started directly from the ancestor (ANOVA; population subsamples vs. ancestor: $P < 0.001$; clones vs. ancestor: $P < 0.001$), but were not significantly different from each other (ANOVA; $P = 0.36$) (Fig. 2). These differences reflect that the glucose-evolved populations overcame an initially lower fitness to reach a higher fitness than the populations started directly from the ancestor. After 1000 generations of evolution, the glucose-evolved populations still had higher total fitness increases than did populations started from the ancestor. At this time point, however, the final fitness of populations started from founder clones was marginally nonsignificantly higher than the fitness of populations started from the ancestor (final fitness: population subsamples vs. ancestor, $P = 0.003$; clones vs. ancestor, $P = 0.086$). Again, the fitness of replay populations started from clones and from population subsamples were not significantly different from one another (ANOVA; $P = 0.27$).

We focused on the six founder glucose-evolved clones to begin to examine a possible basis for their higher evolvability in lactose. A candidate explanation is that the glucose-evolved clones had a higher genomic mutation rate than did the ancestral strain. Higher genomic mutation rates have been associated with more rapid fitness improvement in several laboratory-evolution experiments (Chao and Cox 1983; de Visser et al. 1999). To test this, we used fluctuation tests to measure the spontaneous mutation rate to rifampicin resistance of the glucose-evolved clones and the ancestral strain. We found no substantial difference in this rate, with all ancestor versus glucose-evolved clone mutation rate estimates being within a factor of three of one another (Fig. 3).

A similar mutation rate between founder clones and the ancestor indicates that selection in glucose led to accumulation of mutations that increased lactose evolvability directly. To identify candidate mutational changes that might contribute to evolvability, we sequenced the six founder clones. Because the increased lactose evolvability trait was common to all clones, judged by their similar fitness increases following selection in lactose, we reasoned that mutations that occurred in the same genes in multiple clones would be candidates for contributing to lactose evolvability. We identified three to seven mutations per clone with four genes or gene regions being mutated in at least four clones; *rbs*, *spoT*, *iclR*, and *nadR* (Table 1). These genes are candidates for affecting evolvability, although a determination of their influence will require their removal from founder strains and measurement of the evolvability of those derived strains.

Discussion

Several experiments have found that evolutionary history can influence subsequent evolutionary potential (Travisano et al. 1995; Burch and Chao 2000; Buckling et al. 2003; Cuevas et al. 2009; Barrick et al. 2010; Woods et al. 2011; Perfeito et al. 2013; Kryazhimskiy et al. 2014). In general, this dependence reflects the pervasive effect of epistasis in making the influence of new beneficial mutations dependent on a particular genetic background. For example, to the extent that there is a generally antagonistic relationship between beneficial mutations, initially low fitness genotypes are expected to adapt more quickly than fitter genotypes (Chou et al. 2011; Khan et al. 2011; Wiser et al. 2013; Kryazhimskiy et al. 2014). Indeed, Kryazhimskiy et al. (2014) found that differences in the effect of evolutionary history on

Table 1. Mutations in founder glucose-evolved clones.

	Founder clone					
Mutated gene/region	1	2	3	4	5	6
bioA			G150S			
ECB510/nohB			−144/−245[1]			
fabG/acp	+156/−55[1]					
iclR		T60P	G219R		−58/−142	Δ48
nadR	::IS*150*	S178L	::IS*186*		+4	L289P
pykF				D336N		
rbs	Δ4398	Δ4649	Δ6390	Δ6213	Δ290	Δ3252
spoT		G207D	Δ1	F409V	R701Q	R209L
wecF	N206D					
ybbN		::IS*150*				
ycjX						S294R
yijC		::IS*150*				
yjeP						Δ36
YoaD		G367G				

[1]For intergenic mutations, numbers indicate bases upstream/downstream of adjacent genes. For coding sequence mutations residue and change in one-letter amino acid code, insertion/deletion size, or identity of IS insertion are given.

adaptive potential could be explained mainly through the effect of differences in initial fitness rather than the different genotypes that are the specific basis of those fitness differences.

We found that despite ancestral and glucose-evolved populations having similar initial fitness in lactose, glucose-evolved populations increased in fitness more quickly and to higher levels than the ancestral genotype. Clones derived from the glucose-evolved populations were also more evolvable than the ancestor, indicating that increased evolvability was a consequence of specific mutations, not just of initially higher within-population genetic variability. We cannot distinguish between these mutations being selected to provide a benefit to the glucose component of the environment, or, perhaps to other components, for example, the particular growth regime (oxygenation and pH levels, etc.) prevailing during selection. This result is distinct from some previous studies that have found populations with different evolutionary histories, but with the same or unknown selective histories, to have different responses to subsequent selection (Burch and Chao 2000; Woods et al. 2011). Here, we find that the difference in evolvability is associated with a difference in selective history.

The significant trend of independent glucose-evolved populations being more evolvable than the ancestor indicates that the genetic basis of higher lactose evolvability is a common outcome of evolution in glucose. To explain this, we suggest that increased evolvability could be due to the availability of compensatory mutations that reverse the effects of mutations accumulating during evolution in the glucose environment that are deleterious in the lactose environment. As part of the experiment reported here, we also selected populations derived from the ancestral strain in a constant lactose environment (Cooper and Lenski 2010; Satterwhite and Cooper 2015). We found mutations in *rbs* and *spoT* in six of six, and mutations in *nadR* in five of six, of these populations, suggesting that mutations in these genes confer a benefit in lactose. Indeed, a *rbs* deletion allele and a *spoT* allele isolated previously (Khan et al. 2011) were found by themselves to confer significant fitness benefits in the lactose environment, as well as the glucose environment in which they were selected (*rbs*: glucose 3.2%, $P < 0.001$, lactose 5.2%, $P < 0.001$; *spoT*: glucose 10.1%, $P < 0.001$, lactose 5.6%, $P = 0.002$). Although it is possible that some evolved *rbs*, *spoT,* and *nadR* alleles have glucose-specific benefits, we think a more likely explanation for our observation that the glucose-evolved founder populations had not increased in fitness in lactose is that they accumulated a mix of mutations with beneficial and deleterious effects in the lactose environment that effectively cancel out each other's effects. Candidates for lactose deleterious mutations are those in *iclR*, which were found in five of six glucose but zero of six lactose-selected populations. During selection in lactose, any mutations that arose and compensated for the deleterious mutations would therefore "reveal" the preexisting beneficial mutations. Under this scenario, higher evolvability could result if compensatory mutations occurred at a higher rate than generally beneficial mutations of similar effect or if single mutations could confer very large benefits by compensating large-effect deleterious mutations (Moore et al. 2000; Barrick et al. 2010; Perfeito et al. 2013). In summary, while the genetic mechanism(s) of increased lactose evolvability remains unknown, what is clear is that adaptation to the glucose evolution environment moved genotypes to regions of genetic space where mutations that increased fitness in lactose were either more common or of higher benefit.

Selection in glucose consistently leads to changes in lactose evolvability through accumulation of specific mutations that change the effect of additional mutations. Our results demonstrate that even when adaptation to one environment confers no immediate benefit, or even a small cost, in a second environment, it can nevertheless confer a longer term benefit. We emphasize that fitness in lactose had no direct benefit to the glucose-evolved populations prior to the switch to lactose as the limiting sugar in their selective environment at 2000 generations. The increase in evolvability, while perhaps a consequence of mutations that increased direct fitness, was not itself selected. Of course, whether such a benefit will be realized will depend on the specific circumstances of environmental change.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.16qg8.

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