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Evolutionary Adaptation to Freeze-Thaw-Growth Cycles in *Escherichia coli*

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ABSTRACT

Fifteen populations of *Escherichia coli* were propagated for 150 freeze-thaw-growth (FTG) cycles in order to study the phenotypic and genetic changes that evolve under these stressful conditions. Here we present the phenotypic differences between the evolved lines and their progenitors as measured by competition experiments and growth curves. Three FTG lines evolved from an ancestral strain that was previously used to start a long-term evolution experiment, while the other 12 FTG lines are derived from clones that had previously evolved for 20,000 generations at constant 37°C. Competition experiments indicate that the former FTG group improved their mean fitness under the FTG regime by about 90% relative to their progenitor, while the latter FTG group gained on average about 60% relative to their own progenitors. These increases in fitness result from both improved survival during freezing and thawing and more rapid recovery to initiate exponential growth after thawing. This shorter lag phase is specific to recovery after freezing and thawing. Future work will seek to identify the mutations responsible for evolutionary adaptation to the FTG environment and use them to explore the physiological mechanisms that allow increased survival and more rapid recovery.

Introduction

Most physiological and genetic studies of adaptation to stressful environments focus on the proximate mechanisms that promote survival and growth. By contrast, research on the evolutionary adaptation of organisms to stressful environments examines how new stress responses evolve or existing stress responses are reshaped by selection. One immediate question is whether a population can survive the stress and persist on an evolutionary timescale or whether it will become extinct, and many factors influence the outcome: the frequency of stress conditions, the conditions for growth between bouts of stress, and so on. Assuming that a population survives, then there exists the potential for evolutionary changes that improve survival and recovery under the same or similar stresses in the future.

The direct study of evolutionary changes can be accomplished by observing populations of bacteria or other organisms with suitably short generations while they are propagated in controlled and reproducible laboratory environments (Rose 1984; Lenski et al. 1991; Bennett et al. 1992; Bennett and Lenski 1999; Wichman et al. 1999, 2000; Elena and Lenski 2003; Lenski 2004; Riehle et al. 2005; Chippindale 2006; Herring et al. 2006; Leu and Murray 2006; Schoustra et al. 2006; Zeyl 2006). Advantages of using bacteria in experimental evolution include their rapid generations and large populations as well the capabilities of establishing replicate populations from the same ancestral clone and reviving ancestral and derived cells stored at different times in an experiment. Also, bacteria reproduce asexually, and thus, stable genetic markers can be used to distinguish ancestral from derived genotypes during competition experiments to measure their relative fitness. Moreover, a wealth of genetic, biochemical, and physiological information exists for model species, including *Escherichia coli*, that can enrich and inform analyses built around evolution experiments.

In a long-term evolution experiment, Lenski and colleagues studied the dynamics of phenotypic and genomic evolution in 12 initially identical populations of *E. coli* while they were propagated in a glucose-supplemented minimal medium at 37°C for more than 20,000 generations (Lenski et al. 1991; Lenski and Travisano 1994; Cooper and Lenski 2000; Rozen and Lenski 2000; Schneider et al. 2000; Cooper et al. 2003; Lenski et al. 2003; Pelosi et al. 2006; Woods et al. 2006). Bennett and colleagues performed related experiments in which bacteria derived from this long-term study were used to establish several sets of new populations that continued to evolve in the same medium but under different thermal regimes, including constant lower and higher temperatures as well as in temporally fluctuating environments (Bennett et al. 1992; Lenski and Bennett 1993; Leroi et al. 1994; Travisano et al. 1995; Mongold et al. 1996, 1999; Bennett and Lenski 1999; Cullum et al. 2001; Riehle et al. 2001, 2003, 2005). The latter studies have shown adaptation that is often temperature specific, with frequent but not universal trade-offs in performance at other temperatures.

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There is evidence from these studies, as well as others, to suggest that rates of evolutionary adaptation to stressful environments may often exceed corresponding rates in nonstressful environments (Parsons 1987; Hoffman and Parsons 1993; Lenski and Bennett 1993; Bennett and Lenski 1997; Rutherford and Lindquist 1998; Queitsch et al. 2002).

In this study, we examine the evolutionary adaptation of *E. coli* populations to a regime of alternating days of freezing without added cryoprotectant at −80°C and thawing at room temperature, followed by growth at the benign temperature of 37°C. In preliminary work, we found that repeated freeze-thaw (FT) cycles cause mortality such that without intervening opportunities for growth, populations become extinct. Therefore, the opportunity for growth every other day allows populations to recover and multiply, including the generation of new mutations that might allow improved survival during or faster recovery following FT cycles. Our aims here are to report the basic experimental design, provide evidence for adaptation to this regime, and determine whether adaptation occurs by improved survival, faster recovery, or both. Also, our study shows how evolutionary history can influence evolutionary changes because the populations were founded by strains that had different histories before this experiment. We will conclude by outlining our future plans to identify the genetic and physiological bases of the adaptations reported here.

Before turning to this evolution experiment, we now provide a brief overview of bacteria in nature that experience freezing and thawing conditions, their physiological responses to freezing and thawing, and a preliminary experiment that we performed to identify the conditions for our evolution experiment. Many different bacterial species live in environments that are subject to freezing and thawing, including Siberian permafrost (Rivkina et al. 2000), arctic tundra (Eriksson et al. 2001), and Antarctic ponds (Mountfort et al. 2003). Some of these bacteria live as parts of mat communities that include diatoms and green algae as well as cyanobacteria. Metabolic activity and growth have been reported for some permafrost bacteria at temperatures as low as −10°C (Bakermans et al. 2003). Comparative evidence suggests that evolutionary adaptation to these FT environments has involved changes in cold shock or cold active proteins (Cloutier et al. 1992; Hebraud and Potier 1999; Siddiqui and Cavicchioli 2006), membrane fluidity (Russell 1990; Hebraud and Potier 1999; Ponder et al. 2005), and enzymes that inhibit or promote ice nucleation (Muryoi et al. 2004; Walker et al. 2006). In general, however, little is known about the timescale over which such changes have occurred, the number of mutations needed to allow survival starting from a FT-sensitive progenitor, whether survival during freezing or recovery on thawing is the more important target of selection, and other such issues that can be explored by experimental evolution.

Freezing and thawing are multifaceted stresses that involve not only low temperature but also potentially lethal intracellular ice formation and dehydration resulting from hyperosmotic shock (Mazur 1984; Gao and Critser 2000). Also, freezing and thawing cause oxidative damage (Cox and Heckly 1973) and can thus be mutagenic (Grecz et al. 1980; Calcott and Gargett 1981). In the face of all these potentially damaging effects, many bacteria nonetheless can protect themselves or otherwise recover under the right circumstances (Calcott 1985). Several variables have been shown to affect whether bacteria can survive freezing and thawing, including nutritional status, growth phase, and cooling rate (Calcott 1985; Gao and Critser 2000). For example, bacteria harvested in stationary phase survive freezing and thawing much better than those harvested during exponential growth (Souzu et al. 1989). Furthermore, preexposure to cold, osmotic, and other stresses can increase subsequent FT survival, presumably owing to changes in levels of particular proteins or other cell components that provide cross tolerance (Thammavongs et al. 1996; Drouin et al. 2000; Panoff et al. 2000).

Various approaches could be pursued to investigate the molecular genetic basis of FT tolerance. The traditional genetic approach screens sets of insertion mutants to identify those that are deficient in FT survival but not in their ability to grow under permissive conditions, presumably owing to knockouts of genes whose products are specifically required for FT survival or recovery. Analyses of changes in mRNA and protein levels might allow the identification of other genes whose expression levels are altered during freezing, thawing, or subsequent recovery. An evolutionary approach, such as the one we have pursued, differs because it allows the study of improvements in the stress response and not just the basis of the tolerance as it presently exists in a particular strain or species. Indeed, there may be many different types of evolutionary adaptations that could improve FT survival and recovery, some of which might involve genes other than those already known to be important for this stress response. By studying the evolution of multiple experimental lines, including ones started with different ancestral strains, one can observe the uniformity or diversity of responses to any given stress. Also, the use of a mesophilic organism such as *E. coli*, instead of a psychrophilic species that is already cold adapted, may increase the opportunity to see new evolutionary adaptations to FT conditions on an experimental timescale. In a study by another group, *Lactobacillus delbrueckii* were found to evolve greater FT tolerance after serial propagation in milk with intermittent freezing and thawing (Monnet et al. 2003).

We performed a pilot experiment to isolate cryotolerant mutants of *E. coli* that could better survive repeated freezing and thawing. The ancestor of the long-term experimental populations was subjected to daily FT cycles without added nutrients, but no viable cells remained after 40 d. Therefore, for this study we introduced a growth phase after the FT cycle to allow populations to recover and, potentially, to evolve. Elsewhere, we have also reported that 12 populations of *E. coli* that evolved

in a minimal salts medium with glucose at 37°C for 20,000 generations all became more sensitive to repeated FT cycles than their ancestor, although the extent of this change varied among the derived lines (Sleight et al. 2006). These findings demonstrate genetic variation in FT tolerance, and they also indicate that some of the mutations responsible for improved fitness in that warm environment are detrimental under FT conditions. Also, because these lineages are preadapted to the growth conditions at 37°C, they are interesting progenitors to start an evolution experiment under the freeze-thaw-growth (FTG) regime. We can compare the adaptation to the FTG regime of the 37°C-evolved lines and their progenitors with our a priori expectations that the 37°C-evolved lines have more scope for improvement with respect to FT cycles but less opportunity to improve further during the growth phase.

Material and Methods

Culture Media and Experimental Preconditioning

The bacterial clones used in our experiments, including those evolved in our experiment as well as their progenitors, are kept in long-term storage at −80°C with glycerol added as a cryoprotectant. However, in the FTG evolution experiment and in our assays of FT survival and relative fitness under the FTG regime, we used medium without added glycerol in order to investigate the evolution of changes in survival and recovery. To ensure that cells were in comparable physiological states at the start of assays of FT survival and competitive fitness, cells were removed from the freezer, inoculated into Luria-Bertani medium independently for each replicate assay, and incubated at 37°C for 24 h; they were then diluted 10,000-fold into Davis minimal medium supplemented with glucose at 25 mg/L (DM25) and incubated at 37°C again for 24 h, at which point these cells were used to start all assays.

Long-Term Evolution Experiment and Bacterial Strains

The long-term evolution experiment at constant 37°C is described in detail elsewhere (Lenski et al. 1991; Lenski 2004). In brief, 12 replicate populations evolved for 20,000 generations (3,000 d) starting from two variants of the same ancestral strain of *E. coli* B. One variant (REL606) cannot grow on arabinose, while the other (REL607) is a spontaneous Ara^+ mutant; six populations were founded from each type. The Ara marker is neutral in the long-term experimental environment (Lenski et al. 1991), which consists of daily 1 : 100 transfers into flasks that contain DM25 and incubation with shaking at 37°C. The dilution and subsequent regrowth allow about 6.6 ($=\log_2 100$) generations/d.

FTG Evolution Experiment

Figure 1 gives an overview of our FTG evolution experiment. The 15 FTG populations include three replicates, designated group A, founded by the Ara^+ variant of the original ancestor, and 12 populations founded by clones sampled from each of the long-term populations at generation 20,000, designated group B. These 15 populations evolved for 150 2-d FTG cycles, which equals some 1,000 generations based on the 100-fold dilution and regrowth in alternating days; in fact, somewhat more generations occurred because additional cell growth also offset death during the FT cycle in alternating days. To start the evolution experiment, 1 mL of stationary phase DM25 culture was transferred into a freezer tube and put in a −80°C freezer for 22.5 h. The tubes were then thawed at room temperature (~22°C) for 1.5 h, after which time the contents were diluted 1 : 100 into fresh DM25 and incubated at 37°C without shaking for 24 h. The use of unshaken tubes, rather than shaken flasks, during the growth phase represents a small departure from the methods used in the long-term evolution experiment. This change allowed us to handle more cultures simultaneously. Any effect on oxygen levels is small, owing to the low sugar concentration used in our experiments and the resulting low population density (about $3–5 \times 10^7$ cells/mL in stationary phase, roughly two orders of magnitude below the density in most research with *E. coli*). In a previous study, there was no discernible difference in relative fitness between shaken flasks and unshaken tubes under conditions similar to those used in our study (Travisano 1997).

Thus, the populations in our evolution experiment experienced cycles of a day of freezing and thawing that alternated

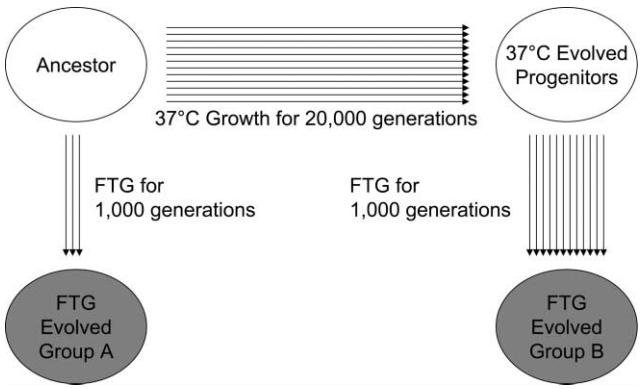

Figure 1. Evolutionary histories of freeze-thaw-growth (FTG) populations. Three lines in FTG-evolved group A were founded by the ancestor of another evolution experiment. Twelve lines in FTG-evolved group B were founded by clones sampled from populations that previously evolved for 20,000 generations at 37°C. All 15 of the FTG lines evolved for 1,000 generations under the FTG regime, with alternating days of a FT cycle and growth at 37°C. The culture medium used for growth days was the same one used during the evolution of the progenitors of FTG group B.

with a day in which they grew in the same medium and at the same temperature as in the long-term evolution experiment. Populations and clones were sampled and stored every 100 generations (30 d) in freezer vials with glycerol for future study. During the long-term evolution experiment at 37°C, unique mutations were substituted at certain loci in each population (Woods et al. 2006); we confirmed by sequencing the presence of at least one such mutation in each group B line, which demonstrates they were all derived from their intended progenitors (S. C. Sleight and R. E. Lenski, unpublished data).

FTG Competition Experiments

We performed competitions to measure the extent of evolutionary adaptation of the FTG-evolved lines relative to their progenitors. Competitions were performed in the exact same environment as used in the FTG evolution experiment. For group A, the FTG-evolved lines competed directly against the ancestor with the opposite Ara marker state. For group B, the FTG-evolved lines and their 37°C-evolved progenitors each competed separately against the original ancestor bearing the opposite marker state.

The densities of two competitors were estimated by serially diluting cultures on tetrazolium-arabinose (TA) indicator plates, except for one FTG-evolved line and its progenitor that form poor colonies on TA medium and whose densities were estimated from minimal glucose plates. Plates were incubated for 24 h at 37°C, and the resulting number of colony-forming units was used to estimate the corresponding density. The Ara^+ and Ara^- cells form white and red colonies, respectively, when grown on TA plates. As a control for any effect of the Ara marker, the Ara^+ and Ara^- ancestral variants also competed in each batch of competition experiments; the Ara marker is neutral in the FTG regime, as was shown under the growth conditions of the long-term experiment.

Before starting a competition, each competitor was separately conditioned as described previously. Equal volumes of each competitor were then mixed, and 1 mL was transferred into a tube and placed in a −80°C freezer. After 22.5 h, the tube was thawed at room temperature for 1.5 h. Then 0.1 mL was diluted into 9.9 mL of DM25 and incubated at 37°C for 24 h. Measurements of the densities of the competitors were taken at the start (before freezing), after thawing, and again after the growth phase that completes the full FTG cycle. From these data, we estimated the relative fitness of the two competitors over the course of the complete FTG cycle by using the initial and final samples, as described next. In addition, we partitioned the overall FTG fitness into two components that reflect FT survival and subsequent growth by using the middle sample, also as described next.

Quantifying FTG Fitness and Its Components

For each replicate competition assay, we first calculated each competitor's realized (net) growth rate r over the 2-d FTG cycle as follows:

$$r = \ln\left(\frac{N_2 \times 100}{N_0}\right),$$

where N_2 is that competitor's final cell density, N_0 is its initial cell density, and the factor of 100 takes into account the 100-fold dilution between the 2 d of the FTG cycle. The overall FTG fitness of one competitor relative to another is simply the ratio of their respective realized growth rates over the complete cycle.

We also calculated each competitor's FT survival s and subsequent growth rate g over the two separate days of the FTG cycle as follows:

$$s = \frac{N_1}{N_0},$$

$$g = \ln\left(\frac{N_2 \times 100}{N_1}\right),$$

where N_1 is the viable cell density measured after the first day of the cycle before the 100-fold dilution. Note that survival is a proportion, whereas growth is a rate. In any case, the relative survival and growth of two competitors are expressed as the ratio of the relevant parameters, so that they are dimensionless quantities, as is the overall FTG fitness.

All these quantities—relative fitness, relative survival, and relative growth rate—were first calculated separately for each replicate assay. We then averaged the values from the replicate assays to obtain the estimate for each line within a group. Finally, for each group, the overall means were calculated by averaging the estimates obtained for each line in the group, except in the case of the single ancestor of group A, where the mean was based on the replicate assays. Details of the statistical analyses are given in "Statistical Methods."

Growth Curves

We obtained separate growth curves, based on optical density (OD), for the evolved clones and their progenitors in order to estimate the durations of their lag phase before commencing growth as well as their doubling times during the growth phase. These growth parameters were first calculated for each line by averaging nine replicate growth trajectories for that line. Group means were then calculated by averaging the estimates for each line in the group, except for the single ancestor of group A whose parameters were estimated directly from the nine replicates. OD measurements were made at the 420-nm wavelength

on 0.2-mL cultures in 96-well microtiter plates incubated at 37°C. The data were collected following either an FT cycle or stationary phase at 37°C, with a comparison between the two curves permitting an estimate of the effect of the FT cycle on the time required to achieve exponential growth following dilution into fresh DM25 medium, as described next.

Calculations of Lag Phase Duration and Doubling Time

Calculations of the duration of lag phase used the general method given by Lenski et al. (1994) with modifications as described here. OD values from growth curve experiments were standardized by dividing by the initial OD (measured immediately after the FT cycle before dilution and divided by 100 to take the dilution factor into account) and $\log_2$ transformed in order to express changes as doublings. The transformed data were then plotted and inspected to identify the window of exponential growth, and linear regression was performed on those exponential-phase data. The doubling time was calculated as the inverse of the slope of the regression. The "apparent" lag phase was then estimated by extrapolating the exponential growth back in time until the regression intersected the initial OD measurement (Fig. 2). Note, however, that this apparent lag phase does not take into account any contribution of dead cells to OD, so it overestimates the actual duration of the lag phase. We corrected for this mortality by independently measuring the FT survival of the clone and then transforming the proportion surviving to a $\log_2$ scale; that proportion is generally below 1, and hence the value is negative on a log scale, with more negative values corresponding to higher mortality. As shown schematically in Figure 2, the $\log_2$-transformed survival value was plotted as a horizontal line on the graphs showing the exponential-phase growth data, where the intersection of this line with the extrapolated regression then provides an estimate of the actual duration of the physiological lag (i.e., the duration corrected for FT mortality).

Statistical Methods

We performed *t*-tests to compare performance measures between the FTG-evolved lines and their progenitors. Paired tests were always used when comparing B group–evolved lines with their progenitors, owing to their unique relationships. Paired tests were also used to compare the A group–evolved lines with the common ancestor when the relevant assays for each evolved line were paired with particular assays for the ancestor. Otherwise, we used a *t*-test for comparing a single specimen, the ancestor, with a sample, the three evolved group A lines (Sokal and Rohlf 1981, pp. 229–231). This test assumes equal standard deviations for the distributions from which the single specimen and the sample are drawn; in fact, the ancestor, being a homogenous type, should have a lower standard deviation than the independently derived lines, which makes our inferences

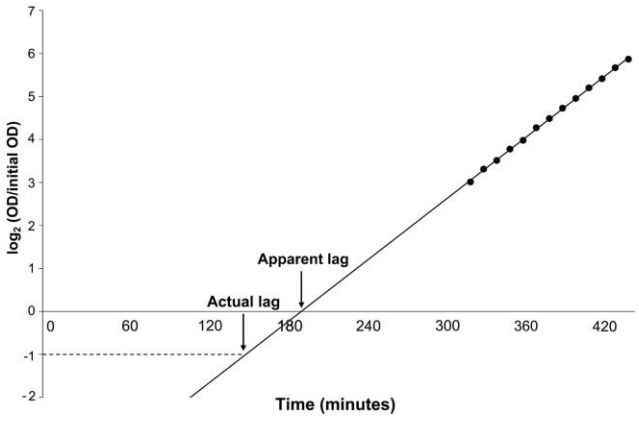

Figure 2. Schematic illustration showing how the apparent duration of lag phase is corrected for freeze-thaw (FT) survival to calculate the actual duration of the physiological lag before growth. The apparent duration is calculated by extrapolating the regression line based on exponential growth back to the initial optical density. In this hypothetical example, 50% of the cells survived the FT treatment; therefore, the initial density of viable cells is half that implied by the initial optical density ($\log_2 0.5 = -1$). The actual duration of the physiological lag is then obtained by extending the extrapolated growth trajectory until it intersects the initial density of viable cells.

conservative. We used one-tailed tests for those hypotheses with clear a priori expectations about the direction of the outcome; otherwise, two-tailed tests were used conservatively, including those cases with potentially opposing effects. For example, we expect overall fitness and its components to increase for all FTG-evolved lines when measured in the FTG environment. We also expect the lines in group B, which previously evolved in the same medium at 37°C and suffered correlated losses in FT survival, to show less improvement in growth rate but greater gains in FT survival than the lines in group A.

Results

Evolutionary Adaptation to the FTG Regime

Figure 3 shows that the lines evolved under the FTG regime improved in their overall fitness when measured under that same regime. The three lines in group A increased their fitness, on average, by 89% relative to the ancestral strain. The 12 lines in group B improved relative to their 37°C-evolved progenitors by 60%, on average. Notice that the progenitors of group B were themselves much more fit than the common ancestor under the FTG regime; this result indicates that their improvements in growth during 20,000 generations at 37°C more than offset their losses in FT survival. The fitness gains in groups A and B relative to their own progenitors were both significant (one-tailed paired *t*-tests: group A, df = 2, $P = 0.0034$; group B, df = 11, $P < 0.0001$). Also, the proportional gains in group A, relative to their ancestor, were significantly greater than those observed in group B, relative to their progenitors, during the

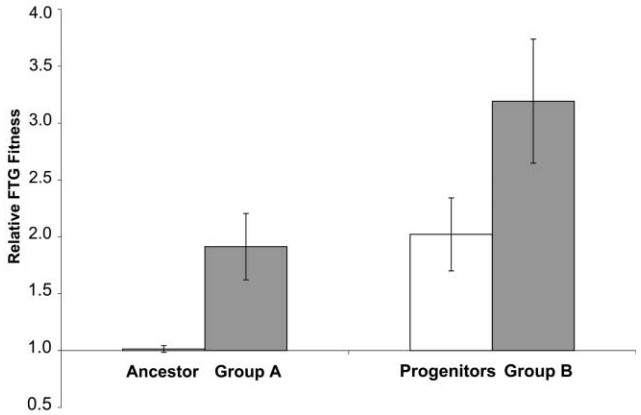

Figure 3. Relative fitness of freeze-thaw-growth (FTG)-evolved groups A and B and their progenitors over the 2-d FTG cycle. For group A, each of the three evolved lines competed against the ancestor with the opposite Ara marker state. For group B, each of the 12 evolved lines and their progenitors competed separately against the original ancestor with the opposite marker state. The mean value for each evolved group (*gray*) is adjacent to the corresponding mean for its progenitors (*white*). Means for each group were calculated from the average values for each member in the group, except for the single ancestor of group A, where the mean is based on six independent measurements. Error bars are 95% confidence intervals based on the number of independently evolved lines, except for the ancestor, for which the interval is based on the six replicate assays.

FTG evolution (two-tailed *t*-test with unequal variances, df = 13, $P = 0.0417$), although B had a final mean fitness that was significantly higher than that of A (two-tailed *t*-test with unequal variances, df = 13, $P = 0.0003$).

Figure 4 illustrates the extent of variation among the 15 evolved lines and their progenitors in overall fitness under the FTG regime. The three group A lines improved to a similar extent, and their fitness gains were all highly significant ($P < 0.0001$, based on one-tailed *t*-tests comparing six replicate assays for an evolved line and two assays for the ancestor that were paired with each evolved line). The group B lines showed much more variability, reflecting differences in their progenitors' fitness as well as their subsequent gains. Eleven of the B lines improved significantly (all $P < 0.05$, based on one-tailed *t*-tests comparing six replicate assays for each evolved line with six replicate assays for its own progenitor), while the gain in line A + 5 was not significant. The A + 5 progenitor had the highest FT survival of all group B progenitors; therefore, it had less scope to improve in this respect.

The fitness values for the B progenitors, which previously evolved at constant 37°C for 20,000 generations, vary by about twofold under the FTG regime (Fig. 4), whereas they vary much less, although significantly, in the benign environment where they evolved (Lenski et al. 1991; Lenski and Travisano 1994; Cooper and Lenski 2000). There is no significant correlation between the FTG fitness values measured here and the fitness values measured previously on the same generational samples (Cooper and Lenski 2000) in that benign environment ($r = -0.1673$, df = 10, two-tailed $P = 0.6033$). Although the medium per se is the same, the ancestral and FTG environments are so dissimilar overall that performance in one does not predict performance in the other. However, we recently showed that while all the B progenitors have greater sensitivity to FT mortality than the common ancestor, the extent of their increased sensitivity was significantly correlated with their fitness gains in the benign environment (Sleight et al. 2006). Thus, prior adaptation by the B progenitors reduced the scope for adaptation to the growth period of the FTG regime while increasing potential adaptation to the FT treatment.

The overall adaptation to the FTG regime can be partitioned into improvements in FT survival and subsequent growth performance. Figure 5*A* shows the changes in FT survival. Both group A and group B lines survived freezing and thawing better than their progenitors (one-tailed paired *t*-tests: group A, df = 2, $P = 0.0014$; group B, df = 11, $P = 0.0023$). The improvement in survival was significantly greater in group B than in group A (one-tailed *t*-test with unequal variances, df = 13, $P = 0.0432$), as expected given the higher initial mortality in B progenitors. The resulting final survival values did not differ significantly between groups A and B (two-tailed *t*-test with unequal variances, df = 13, $P = 0.3691$). Figure 5*B* shows the corresponding changes in growth performance after an FT cycle and dilution into fresh medium. Both groups underwent sig-

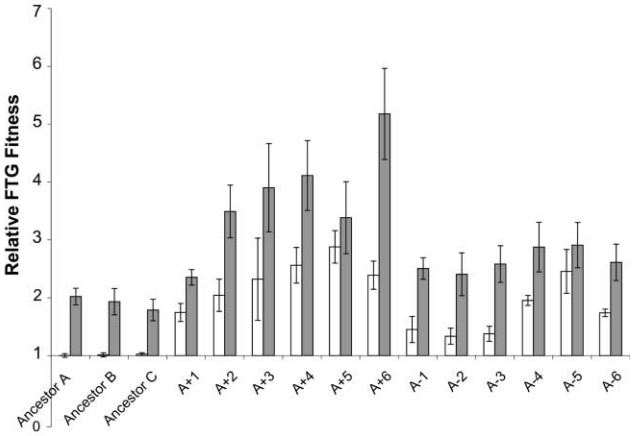

Figure 4. Relative fitness of the 15 freeze-thaw-growth (FTG)-evolved lines and their progenitors. Fitness values were measured in competition with the common ancestor over the 2-d FTG cycle. Each evolved line (*gray*) is paired with its progenitor (*white*). The three group A lines are shown with different ancestral replicates (designated A, B, and C). The 12 group B lines were derived from long-term lines, designated A + 1 to A − 6, that previously evolved for 20,000 generations at 37°C. Error bars show 95% confidence intervals based on six replicate fitness assays for each line or progenitor, except for the group A ancestor, where two of the replicate assays were paired with each of the three evolved lines in that group.

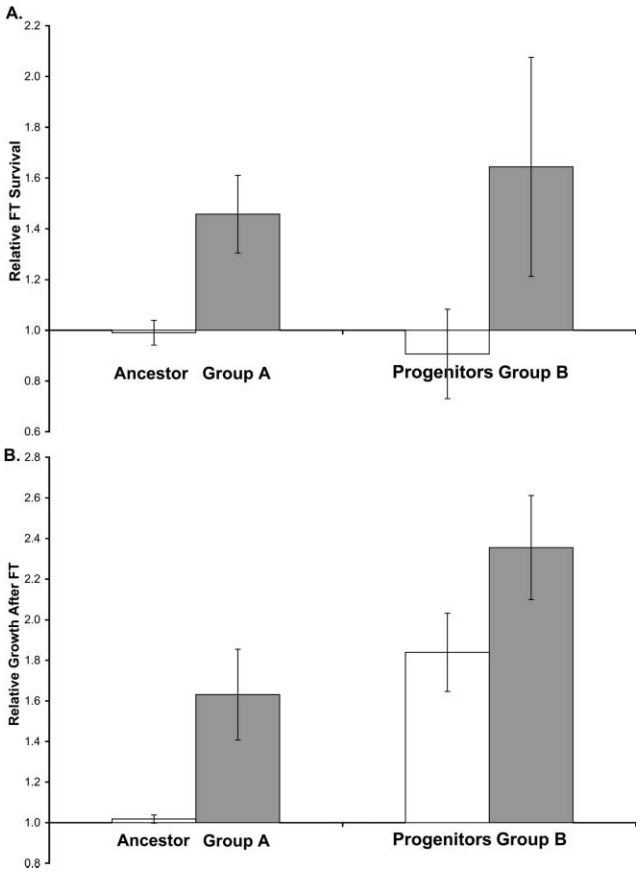

Figure 5. Decomposition of overall fitness gains under the freeze-thaw-growth (FTG) regime into changes in freeze-thaw (FT) survival (*A*) and growth performance (*B*). All values were measured with, and are shown relative to, a marked variant of the common ancestor. FT survival reflects changes in viable cell density during the first day of FTG competitions. Growth performance reflects the net rate of increase on the second day after dilution into fresh medium. The mean for each evolved group (*gray*) is adjacent to the corresponding mean for its progenitors (*white*). Means for each group were calculated from average values for each member in the group, except for the single ancestor of group A where the mean is based on six independent measurements. Error bars are 95% confidence intervals based on the number of independently evolved lines, except for the ancestor, for which the interval is based on the six replicate assays.

nificant improvement (one-tailed paired *t*-tests: group A, df = 2, $P = 0.0042$; group B, df = 11, $P < 0.0001$). The proportional gain in growth performance was greater for group A than for group B (one-tailed *t*-test with unequal variances, df = 13, $P = 0.0056$), as expected given the higher initial level for the group B progenitors. However, the final growth performance was still higher in B than in A (two-tailed *t*-test with unequal variances, df = 13, $P = 0.0001$).

The fact that group B lines showed such large improvements in their growth performance in the second day of the FTG cycle, despite their progenitors having evolved at 37°C for 20,000 generations, may be surprising at first glance. However, when we performed 1-d competitions in the same medium without an FT cycle, the evolved group B lines did not show any improvement in growth performance (S. C. Sleight and R. E. Lenski, unpublished data). As we will show next, their improvement does not come from faster rates of exponential growth, but instead they evolved faster recovery of their growth capacity following the FT treatment.

Changes in Growth Dynamics Including the Duration of Lag Phase

Figure 6 shows the growth dynamics, based on OD, for both FTG-evolved groups and their progenitors under two slightly different conditions. In Figure 6*A*, the growth data correspond precisely to day 2 of the FTG regime, with cells having been frozen and thawed before their dilution into fresh medium at 37°C. In Figure 6*B*, the populations were started from stationary-phase cultures grown at 37°C without the intervening FT cycle.

Several interesting features are apparent from the growth dynamics following the FT cycle (Fig. 6*A*). First, notice that both evolved groups (*gray curves*) increase much sooner than their respective progenitors (*black curves*). Second, the group B–evolved lines (*solid gray curve*) perform better, on average, than the group A lines (*dashed gray curve*), although the difference is much less than between their progenitors (*solid black*, *dashed black curves*, respectively). Third, the improvement in group A (*dashed gray curve*) relative to their ancestor (*dashed black curve*) appears to involve both earlier and faster growth.

Now compare these dynamics with those observed without the intervening FT treatment (Fig. 6*B*). First, notice that all four growth trajectories now rise earlier than before. These differences imply that some portion of the lags after the FT treatment reflect demographic recovery, physiological recovery, or both from that stress. Second, the trajectories for the group B lines and their progenitors (*solid gray*, *solid black curves*, respectively) are almost perfectly superimposed. This similarity, in contrast to the difference seen after the FT treatment, implies that the group B lines have adapted by accelerating their recovery from the stress. Third, the trajectories for group A and the ancestor (*dashed gray*, *dashed black curves*, respectively) are much closer than they were following the FT treatment. The remaining difference between them appears to reflect faster growth by the evolved A lines rather than a shorter lag phase. After quantifying these differences, we will turn in the next subsection to more intensive experiments to disentangle the roles of demographic and physiological recovery from the FT treatment.

For each FTG-evolved line and its progenitor, we calculated its doubling time during exponential-phase growth and the "apparent" lag duration (without adjusting for FT mortality), as described in "Material and Methods." Considering first the

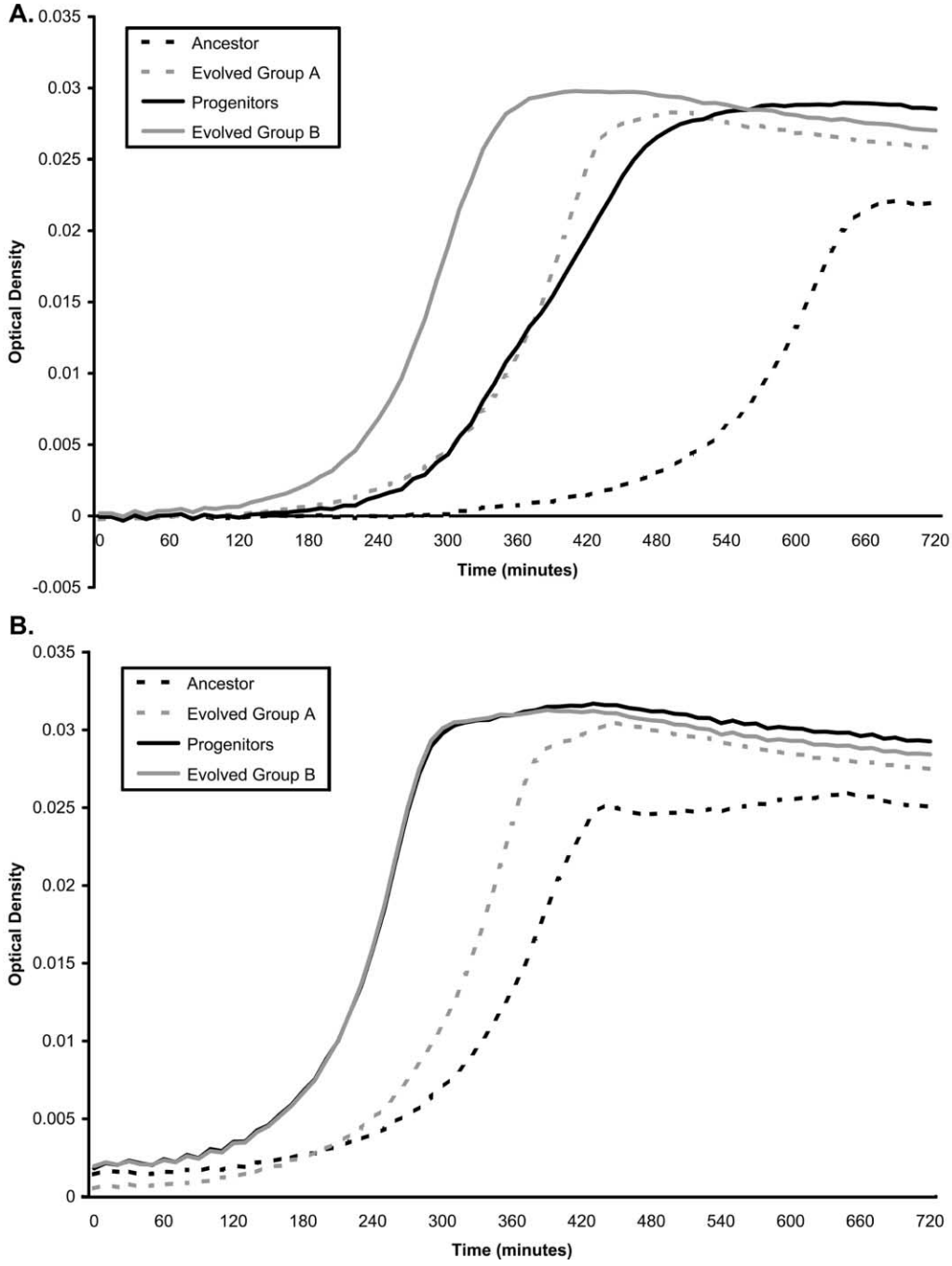

Figure 6. Growth dynamics of the freeze-thaw-growth–evolved lines and their progenitors following freeze-thaw treatment (*A*) and stationary phase at 37°C (*B*). Evolved group A and the ancestor are shown by dashed gray and dashed black curves, respectively. Evolved group B and progenitors are shown as solid gray and solid black curves, respectively. Each trajectory shows the mean calculated over all of the evolved lines or progenitors in a group, except for the single group A ancestor. In each case, the underlying trajectories were replicated ninefold for each evolved line or progenitor, including the group A ancestor.

growth trajectories after the FT treatment (Fig. 6*A*), group A lines evolved much faster exponential growth rates, with their mean doubling time almost 13 min shorter than that of the ancestor (one-tailed $P = 0.0075$ using a *t*-test for comparing a single specimen, the ancestor, with the three evolved group A lines). The group A lines also dramatically shortened the mean duration of the apparent lag phase by 153 min relative to their ancestor (one-tailed $P = 0.0066$, same test as above). By contrast, the group B lines, on average, did not evolve faster exponential growth than their progenitors, with the trend slightly in the opposite direction ($P > 0.5$). However, the B lines also evolved a much faster transition into exponential growth, with the average duration of the apparent lag phase reduced by about 105 min (one-tailed paired *t*-test, df = 11, $P = 0.0001$).

Without the preceding FT cycle (Fig. 6*B*), group A lines again showed faster exponential growth rates relative to those of their progenitors, with the average reduction in doubling time estimated to be about 9 min from these data (one-tailed $P = 0.0058$ using a *t*-test for comparing a single specimen with a group). However, the A lines did not evolve any shorter lag phase under this treatment, with the observed trend in the opposite direction ($P > 0.5$). Relative to their immediate progenitors, the group B lines did not improve, on average, in either their doubling time (one-tailed paired *t*-test, df = 11, $P = 0.3007$) or the duration of the lag phase before growth, which trended very slightly in the opposite direction ($P > 0.5$).

Demographic and Physiological Contributions to Shorter Lag Phases

The data in the previous subsection clearly show that the FTG-evolved bacteria evolved much shorter apparent lag phases and that these changes were specific to the FT treatment. However, these reductions in apparent lag phase do not distinguish between reduced cell death, such that population growth recommenced from a higher level after the FT treatment, and faster physiological recovery of surviving cells. To distinguish between these two effects, one must adjust the apparent lag phase by taking into account mortality during the FT treatment. Recall Figure 2, which shows schematically how the actual duration of the physiological lag phase is corrected for this FT mortality. Whereas the apparent lag is calculated by extrapolating exponential growth back to the initial OD, which includes both living and dead cells, the actual lag is calculated by extrapolating back to the density of surviving cells only.

To address this issue, we performed more intensive experiments, including growth trajectories and FT survival assays, for single representatives of each evolved group and their progenitors. For group A, we used the middle of the three evolved lines and the common ancestor; for group B, we used the evolved line designated as A + 1 and its progenitor (Fig. 4). Figure 7*A* shows the estimation of the duration of the physiological lag for that group A–evolved line and its ancestor. The ancestor (*black symbols*) had an apparent lag of 324 min, while the apparent lag for the evolved line (*gray symbols*) was only 155 min, the difference being 169 min. The physiological lags, adjusted for cell mortality, were 282 min for the ancestor and 135 min for the evolved line. Thus, the physiological lag was reduced by 147 min during the evolution of this FTG-adapted line. The other 22 min of the difference in the duration of the apparent lag reflects improved survival during the FT treatment.

Figure 7*B* shows the same analysis for the group B–evolved line and its own immediate progenitor, which evolved previously at 37°C for 20,000 generations. The apparent lag for this evolved line was 76 min, while that of its progenitor was 192 min, with the duration reduced during FTG evolution by 116 min. After adjusting for mortality during freezing and thawing, the physiological lags estimated for the evolved line and its progenitor were 56 and 148 min, respectively, indicating that evolution had reduced the physiological lag by about 92 min in this group B line.

Figure 8 shows the calculation of lag durations for the same evolved lines and their progenitors, except in this case without an FT treatment before growth. There were no adjustments for death, which does not occur at any measurable rate under these conditions (Vasi et al. 1994). For both evolved lines and their progenitors, lag phase durations were shorter than those seen after an FT treatment. Moreover, the differences between the FTG lines and their progenitors were much smaller, and in the group A case, the order was reversed. Without an FT treatment, the group A–evolved line had a lag duration of 84 min, whereas that of the ancestor was only 52 min (Fig. 8*A*). Notice, too, that this group A line evolved faster exponential-phase growth, consistent with the shorter doubling times reported earlier for its group. The group B line showed no improvement in exponential growth rate, as expected given its progenitor's prior evolution in the same medium and again consistent with earlier results (Fig. 8*B*). This group B progenitor experienced a lag of 34 min without an FT treatment, while its FTG-evolved derivative had a lag of 6 min. The resulting difference of 28 min is much less than the 92-min difference in lag duration observed for the same B line and its progenitor after the FT treatment (Fig. 7*B*).

These experiments demonstrate that greater survival during the FT treatment and faster physiological recovery of growth capacity both contributed to the improved fitness of lines that evolved under the FTG regime. We obtained additional evidence for the evolution of faster physiological recovery in two subsidiary experiments (data not shown). First, we obtained some population growth trajectories by plating and counting cells rather than by combining measurements of ODs and cell survival. These experiments showed the faster recovery of growth in the same two evolved lines relative to their progenitors. Second, we performed fluorescence microscopy at multiple times after an FT cycle in order to visualize actively di-

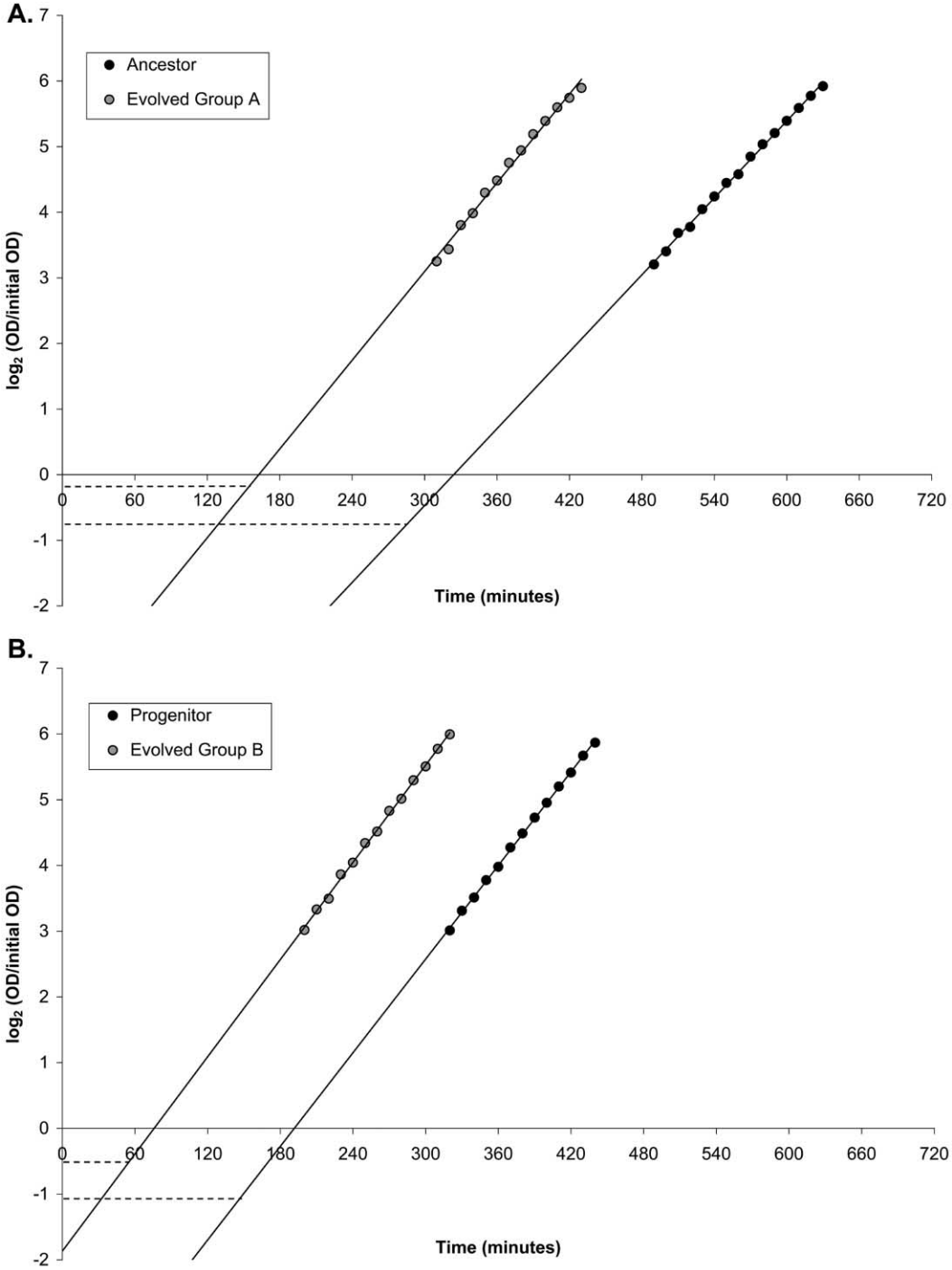

Figure 7. Lag phases following the freeze-that treatment for two freeze-thaw-growth–evolved lines and their progenitors. *A*, group A–evolved line and the ancestor; *B*, group B–evolved line and its immediate progenitor. The evolved lines are shown as gray symbols and their progenitors as black symbols. The apparent duration of the lag phase is where the log-linear regression crosses the initial optical density (OD). The actual physiological duration occurs when the regression crosses the density adjusted for the proportion of surviving cells in the initial population, shown by the dashed horizontal lines. Growth trajectories and survival calculations are means of 27 replicate assays for each evolved line and progenitor.

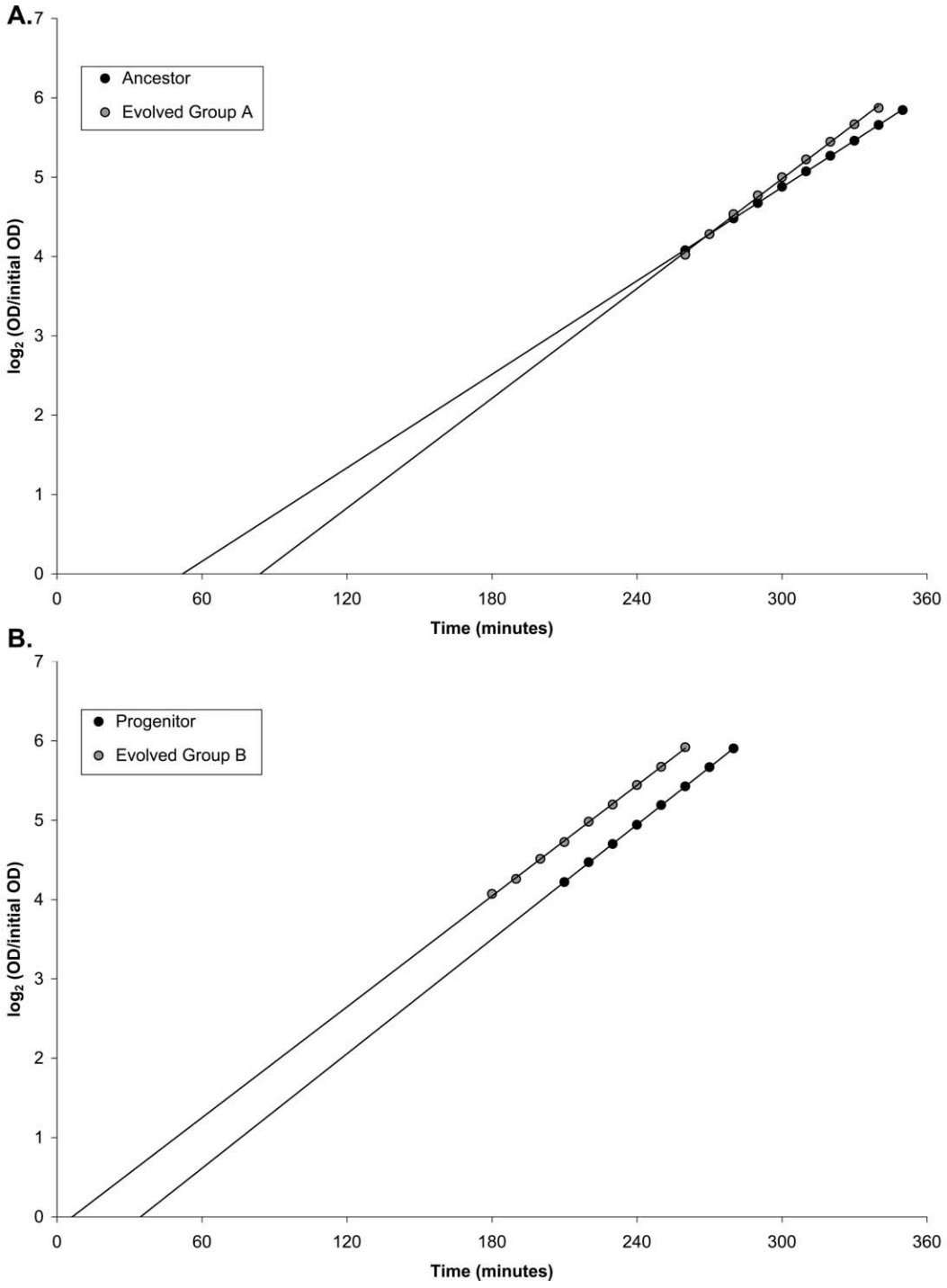

Figure 8. Lag phases following stationary phase at 37°C for two freeze-thaw-growth–evolved lines and their progenitors. *A*, group A–evolved line and the ancestor; *B*, group B–evolved line and its immediate progenitor. The evolved lines are shown as gray symbols and their progenitors as black symbols. The duration of the lag phase extends to where the log-linear regression crosses the initial optical density (OD); there is no appreciable mortality under this regime. Growth trajectories are means of 27 replicate assays for each evolved line and progenitor.

viding cells. These observations revealed that cell division began much earlier in the FTG-evolved lines than in their progenitors, confirming the evolution of faster physiological recovery of growth capacity following this stressful treatment.

Discussion

We performed an experiment to investigate the evolutionary adaptation of 15 *E. coli* populations to 150 2-d cycles of freezing, thawing, and growth. No glycerol or other cryoprotective compound was added before freezing, unlike the standard method used to store bacteria. The entire experiment encompassed more than 1,000 generations. Twelve of these FTG lines were founded by progenitors that had previously evolved for 20,000 generations in the same medium and at the same growth temperature of 37°C, except without the FT treatment between reaching stationary phase and dilution in fresh medium (Fig. 1). The 37°C-evolved progenitors had higher growth rates than did the ancestor of the other three FTG lines, but these 37°C-evolved progenitors also suffered greater mortality during the FT treatment.

Almost all of the evolved lines showed large fitness gains in the FTG regime (Figs. 3, 4). We identified three distinct fitness components that contributed to the improvement in overall performance. First, both groups of the evolved lines exhibited substantial improvement in FT survival, although the magnitude of improvement was greater for the group B lines, whose progenitors had evolved in the same medium at constant 37°C (Fig. 5*A*). During evolution in that benign environment, the progenitors became more sensitive to FT mortality (Sleight et al. 2006); hence, there was greater opportunity for better survival, and indeed this occurred. Second, the group A lines, whose progenitors had not experienced the same medium, evolved faster exponential-phase growth (Figs. 6–8). By contrast, the group B lines showed no measurable gains in their exponential growth (Figs. 6–8). Evidently, the B progenitors had largely exhausted the potential for faster exponential growth rate during their 20,000-generation history in the same culture medium.

Third, and perhaps most interesting, both groups showed striking improvement in the speed of their physiological recovery from FT stress. To demonstrate and quantify this third component of adaptation, we had to disentangle two confounding effects. One such factor is mortality during the FT cycle, which contributes to delayed recovery at the population level but does not bear directly on the speed of recovery by surviving cells. We addressed this issue by independently measuring FT mortality and using the data to calculate the physiological lag (Figs. 2, 7, 8). The second complication arises because physiological lags occur following starvation, even without the FT treatment, on dilution into fresh medium. Thus, one must distinguish between recovery from starvation and from freezing and thawing, which we did by measuring the duration of physiological lags following starvation with and without the intervening FT treatment (Figs. 6–8). Both groups of FTG-evolved lines showed much faster recovery of their surviving cells, with growth commencing 1 h or more sooner than it did in their progenitors. This accelerated recovery provides a substantial head start during the subsequent exponential growth. We also performed fluorescence microscopy on populations while they were recovering from the FT treatment; these observations confirmed that many more viable cells were in the process of doubling for the FTG-evolved lines than for their progenitors during the early recovery period.

In addition to the differences in adaptation between the two groups of evolved lines, substantial variation also exists among lines in the same group, especially in the B group. The differences in adaptation between groups A and B reflect differences in their selective histories, but this explanation cannot explain the striking diversity among the B lines (Fig. 4), which shared the same original ancestor and the same two-stage history (Fig. 1). Evidently, the 12 lines in the B group, by chance, accumulated different sets of mutations that cause the differences in their performance. We do not know the identity of all those mutations. However, we can ask whether the variation among them reflects, at least in part, heterogeneity that was already present in their progenitors that diverged for 20,000 generations at constant 37°C. In fact, there is a significant positive correlation between the FTG fitness levels of the evolved B lines and their progenitors ($r = 0.6769$, $\mathrm{df} = 10$, one-tailed $P = 0.0079$), indicating that some of the differential success among the diverse B lines reflects variation in the extent of preadaptation among their progenitors. However, it is also clear that most or all the B lines underwent substantial evolutionary adaptation during the FTG experiment (Figs. 3, 4). With only one derived line from each progenitor, we cannot explicitly test whether the different B progenitors predisposed different final states (cf. Travisano et al. 1995). However, we might still examine this issue in the future by finding the mutations that contribute to differences among the progenitors, finding other mutations responsible for adaptation during the FTG evolution experiment, and then systematically recombining these mutations from different lineages to ask whether the beneficial effects of the later mutations are conditional on or, alternatively, independent of the earlier mutations.

Many more experiments remain to be done, of course, so we will close by discussing briefly our ongoing research and future directions. In this study, we have demonstrated evolutionary adaptation to the FTG regime, and we have partitioned the improvement into three distinct fitness components—survival, recovery, and growth—but we have not yet identified the physiological, biochemical, and genetic bases of the improvements in organismal performance. One can imagine approaching this general problem by beginning at a physiological scale and working down toward the genetic level, or vice versa. The approach that we are undertaking begins at the genetic level,

which reflects our own expertise as well as the power of molecular genetic analyses in bacteria. Once we have found some of the mutations responsible for the improved performance of the FTG-evolved lines, we can explore the resulting biochemical and physiological mechanisms. By having gene identities in hand, we can then target future studies to particular pathways based on the wealth of information that links the genetics, biochemistry, and physiology of *E. coli* (Böck et al. 2006).

There are several different genetic approaches that one could pursue to find mutations substituted in experimental populations. These approaches include DNA fingerprinting methods to find genes with new insertions or deletions (Papadopoulos et al. 1999; Schneider et al. 2000; Riehle et al. 2001), sequencing candidate genes based on specific phenotypic changes or other prior information (Notley-McRobb and Ferenci 2000; Cooper et al. 2001; Crozat et al. 2005; Maharjan et al. 2006; Woods et al. 2006), analyzing changes in gene-expression profiles to suggest additional candidates (Cooper et al. 2003; Riehle et al. 2003; Pelosi et al. 2006), and even whole-genome sequencing (Shendure et al. 2005; Herring et al. 2006; Velicer et al. 2006).

Finding mutations, although certainly not trivial, is becoming much easier and less costly. But finding mutations is also only a first step in the genetic analysis. An extremely important subsequent step is to manipulate the affected gene, for example, by constructing isogenic strains that differ by a single mutation (Cooper et al. 2001, 2003; Crozat et al. 2005; Pelosi et al. 2006). Such work is possible using powerful molecular genetic approaches available in this system, but it is also painstaking and challenging work. Once such strains have been constructed, they can be competed to test whether a particular mutation did, in fact, contribute to the observed evolutionary adaptation or, alternatively, merely hitchhiked and was inconsequential to any gains in performance. Of course, the same strains can also be used to examine changes in biochemical and physiological traits, and the connections between genotype, phenotype, performance, and fitness can thus be elucidated.

There are many candidate biochemical and physiological pathways by which the FTG-evolved lines might have improved their FT survival as well as their subsequent recovery and transition to growth. Some possibilities are altered induction of stress responses, including molecular chaperones; better repair of damage to DNA or other cell components; and regulatory or structural changes affecting membrane fluidity, osmosis, DNA supercoiling, and ribosomes or other components of the translational machinery. We are proceeding using a two-pronged approach. First, we have chosen a set of six candidate genes of interest because of their known involvement in some of the relevant pathways, and these genes are being sequenced in the FTG-evolved lines and their progenitors. Second, we are taking a genomic-fingerprinting approach to look for insertions and deletions caused by the movement of insertion-sequence elements that are native to *E. coli* genomes (Papadopoulos et al. 1999; Schneider et al. 2000, 2002; Cooper et al. 2001).

Without giving away the results of future articles, we have found two genes in which many FTG-evolved lines have substituted parallel mutations. Such parallelism is a hallmark of adaptive evolution (Wichman et al. 1999; Cooper et al. 2003; Woods et al. 2006). These genes and their products are in two different physiological pathways, one affecting membrane fluidity and the other encoding a stress response. We are currently attempting to construct isogenic strains that differ only by mutations in these genes. We will then use these strains to analyze the effects of the mutations on the relevant pathways as well as on the demographic and physiological components of survival and recovery that produce the evolutionary adaptation to the FTG regime.

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