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DEFINED ORDER OF EVOLUTIONARY ADAPTATIONS: EXPERIMENTAL EVIDENCE

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Organisms often adapt to new conditions by means of beneficial mutations that become fixed in the population. Often, full adaptation requires several different mutations in the same cell, each of which may affect a different aspect of the behavior. Can one predict order in which these mutations become fixed? To address this, we experimentally studied evolution of *Escherichia coli* in a growth medium in which the effects of different adaptations can be easily classified as affecting growth rate or the lag-phase duration. We find that adaptations are fixed in a defined and reproducible order: first reduction of lag phase, and then an increase of the exponential growth rate. A population genetics theory explains this order, and suggests growth conditions in which the order of adaptations is reversed. We experimentally find this order reversal under the predicted conditions. This study supports a view in which the evolutionary path to adaptation in a new environment can be captured by theory and experiment.

KEY WORDS: **Evolutionary adaptation, evolutionary path, experimental evolution, population dynamics.**

Organisms placed in a new environment evolve to increase their fitness, a process known as evolutionary adaptation (Rosen 1967; Hartl et al. 1985; Ibarra et al. 2002; Conant and Wagner 2003; Cooper et al. 2003; Kishony and Leibler 2003; Balagadde et al. 2005; Dekel and Alon 2005; Orr 2005; Alon 2006; Hegreness et al. 2006; Lenski et al. 2006; Desai et al. 2007; Hegreness and Kishony 2007). Often, multiple different beneficial mutations can contribute to this fitness (Cooper et al. 2003; Segre et al. 2006; Desai et al. 2007). The existence of multiple mutations that an organism can acquire on its way to adaptation may result in many possible alternative evolutionary "paths" (Weinreich et al. 2006).

Classical theories (Fisher 1930; Monod 1949; Orr 1998, 2005) explore the case in which adaptation occurs by means of many mutations each of small effect, resulting in a large number of possible paths. More recently, mutational paths have been the focus of theories and experiments on the statistical properties of mutations (Kimura 1983; Elena et al. 1996; Gerrish and Lenski 1998; Orr 1998, 2000; Holder and Bull 2001; Poelwijk et al. 2006; Weinreich et al. 2006; Desai et al. 2007; Poelwijk et al. 2007). Experiments in phages suggested some repeatability in the pathway to adaptation, with high fitness mutations becoming fixed earlier than other mutations (Holder and Bull 2001). Experiments on protein evolution showed that most of the evolutionary paths to higher fitness are inaccessible under many plausible conditions because they encounter negative epistasis (Weinreich et al. 2006; Poelwijk et al. 2007).

Here, we experimentally study the path to adaptation of a well-studied organism, *Escherichia coli*, in a situation in which fitness (mean number of offspring per unit time) is affected by two main factors. The first factor is the exponential growth rate of the cells (Monod 1949). The second factor is the duration of the lag phase (Buchanan and Solberg 1972; Pirt 1975; Swinnen et al. 2004): the amount of time that it takes cells to begin exponential growth after a shift into fresh medium (Ng et al. 1962; Robinson et al. 1998; McMeekin et al. 2002). The experimental design made it possible to separate the adaptations by their effects on these two phenotypic factors. We then analyzed the results using population dynamics models to determine the number, order, and fitness advantage of the mutations that contribute to the adaptations, which occurred over the evolutionary path.

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Results

SERIAL DILUTION EXPERIMENT IN A POOR GROWTH MEDIUM

We grew *E. coli* K12 strain MG1655 in a chemically defined minimal medium (M9 salts) supplemented with succinate, a poor carbon source. The lag phase of the wild-type cells in this medium is about 15 h, and the exponential doubling rate is once every 2.5 h (Fig. 1).

We used serial dilution method (Lenski and Travisano 1994; Cooper et al. 2003; Crozat et al. 2004) to evolve the bacteria in this growth medium over several hundred generations. The bacteria were grown in a shaken tube until stationary phase was reached. Every two days, cells were diluted 100-fold into a tube containing fresh medium. Hence, in every round of serial dilution, the bacteria passed through lag, exponential, and stationary phases of growth. In every round, the cells grew 100-fold, corresponding to $\log_2(100) = 6.6$ generations.

EXPERIMENTS SHOW A DECREASE IN LAG PHASE FOLLOWED BY AN INCREASE IN GROWTH RATE

Growth rate and lag phase of the cells were monitored over the course of evolution, by measuring the growth curves of samples from the tubes in an automated fluorimeter that measured cell density at a temporal resolution of 2 min over more than 24 h of growth. We find that mutants in which the lag phase was dramatically reduced from 15 h to 5 h became fixed in the population after 65 generations (10 rounds of serial transfer) (Fig. 2A). This adaptation had only a minor effect on the exponential growth rate, increasing it by about 8% (Fig. 2B, dotted circle). These mutants began to have a detectable effect on the mean lag duration of the population as early as after 25 generations (four serial transfers). A control experiment, in which we measured the distribution of the wild-type lag-phase duration in colonies isolated after 48 h of growth, shows a distribution with a single peak around 15 h (online Supplementary Fig. S1). This indicates that lag mutants do not exist in large numbers in the wild-type population.

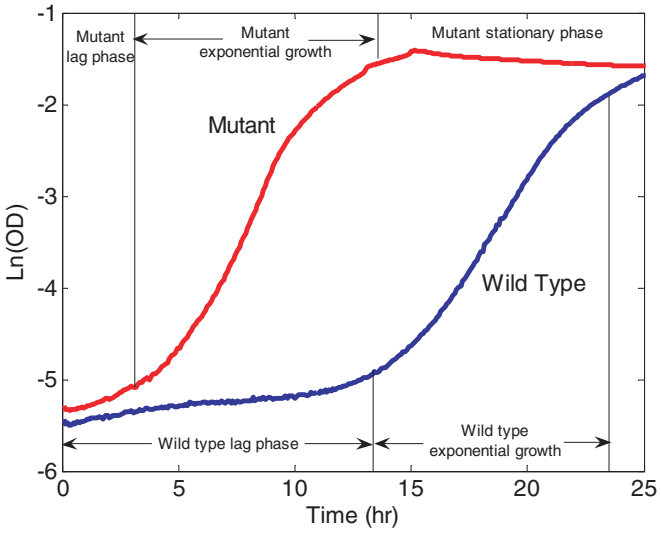

Figure 1. Comparison between the measured growth cycle of wild-type and evolved bacteria on succinate minimal medium. The three growth phases—lag phase, exponential phase, and stationary phase—are indicated. The lag-phase duration of the evolved bacterial population is considerably shorter than the wild type.

After the lag-phase adaptation was established, it took the cells another 150 generations to show a second adaptation: an adaptation that substantially increased the exponential growth rate (Figs. 1 and 2B) but did not affect the lag-phase duration. The overall increase in growth rate was about 35% (doubling time reduced from 2.5 ± 0.2 h to 1.6 ± 0.2 h). The growth rate mutants reached fixation after another 200 generations.

We performed six repeats of the experiment up to 100 generations. The lag phase adapted first and was reduced by the same magnitude, to within experimental accuracy, in all repeats of this experiment.

We also tested the lag phase of the evolved strain with a different carbon source, glycerol. We find that this strain also had a shorter lag than the wild type in the same medium (online Supplementary Fig. S2). This suggests that the adaptation applies to carbon sources beyond the one in which it was selected.

We also compared the survival in stationary phase of the adapted strains to the wild-type cells. We find that in stationary phase cultures (48 h after inoculation), the number of colony-forming units of the wild-type strain is fivefold higher than the evolved strain (online Supplementary Fig. S3). Because a brief lag phase presumably requires maintenance of metabolic enzymes during stationary phase, there is a possibility of a trade-off: reduced lag phase may be linked with reduced survival in stationary phase.

POPULATION DYNAMICS SIMULATIONS SHOW THE SAME EVOLUTIONARY PATH

We next analyzed these results by means of a population dynamics simulation (Hartl and Dykhuizen 1984; Hartl and Clark 1997). The simulation starts with a wild-type population of $N_i = 10^8$ cells. In each simulated round of serial transfer, the cells first do not divide during the lag phase, after which they grow exponentially until their concentration reaches $N_f = 10^{10}$ cells. Then, 1% of the cells are randomly selected to seed the next round of serial transfer (a 100-fold dilution). During growth, mutants of type k occur with a probability p_k per cell per division. The mutations occur randomly in the population.

Let us begin with a simple case in which each adaptation occurs by a single mutation. In this case two kinds of mutations can occur: one that reduces lag phase from 15 h to 5 h and the other that increases growth rate by 35%. In this simple model, there are only

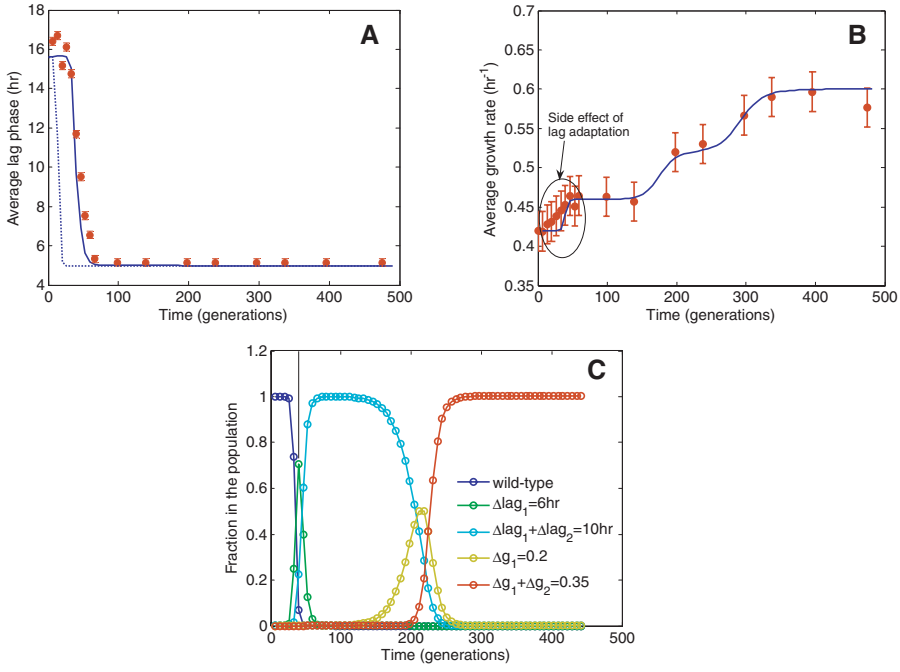

Figure 2. **Experiment and calculation of lag-phase duration and growth rate as a function of the number of generations that *E. coli* evolved in the experiment on succinate minimal medium. (A) Lag-phase duration measurements of cell population (circles). The solid line is the population dynamics simulation results, with two mutations that shortens lag phase by 6 h and 4 h. Dotted line is the best-fit simulation with only a single mutation that shortens lag phase by 10 h. (B) Growth rate measurements of cell population (circles). Blue line is the result of the simulation with two mutations that increase growth rate by 15% and 10%. (C) Calculation of the relative populations of wild type (blue line) and mutants as a function of evolutionary time. The mutational order is seen: the first (two) mutations affect the lag phase (green and cyan), whereas the two growth rate mutations are fixed only after the lag mutants reached fixation. The dotted vertical line indicates the time point where the distribution of the lag-phase durations was experimentally measured (Fig. 3).**

two possible paths that evolution can take. The first path is fixation of the lag-phase mutants, followed by a mutation that increases the growth rate. The second evolutionary path is in the reverse order, first growth rate and then lag phase. As we discuss below, the experiments suggest that there is a small amount of pleiotropy between the lag phase and exponential phase adaptations. This is also included in the present simulations (the lag-phase mutation also increases growth rate by 8%). However, this pleiotropy turns out to have only minor effects on the qualitative results, and one can think of lag phase and growth rate adaptations as separate effects.

The simulations show that although the two kinds of mutants are generated constantly and independently in the population, the lag-phase mutants always take over the population before the growth rate mutants can be observed in these conditions. This deterministic evolutionary path is due to the large advantage conferred in shortening the lag phase compared with the advantage in increasing the growth rate: the mutants with reduced lag phase reach stationary phase before the wild-type cells begin to exit their lag-phase period. Thus, the lag-phase mutants become fixed in the population within two rounds of serial transfer (Fig. 2A, dashed line).

SIMULATION SUGGESTS THAT LAG-PHASE REDUCTION OCCURS BY TWO DISTINCT MUTATIONS

A closer comparison of the experimental data to the simulations indicates that two cumulative mutations, and not one, are needed to explain the observed dynamics of the reduction in lag duration. Assume that the mutational probability of a single mutation in *E. coli* cannot be lower than 5×10^{-10}, it is evident that in the first stage of evolution (up to 100 generations) the observed population dynamics cannot be explained by a single mutation that shorten the lag period from 15 h to 5 h. This is because such a single mutation would become fixed much more rapidly than observed (compare Fig. 2A dashed line to data). The experimental data can be explained by the cumulative effects of two mutations that shorten the lag phase: the first mutation reduces the lag phase by 6 ± 1.5 h, whereas the second reduces the lag phase by another 4 ± 1 h. Cells in which both mutations have accumulated reach fixation after 65 generations (Fig. 2A and 2C). The predicted mutational probabilities are $P = (5 \pm 2) \times 10^{-8}$ and $P = (1 \pm 0.5) \times 10^{-7}$ for the 6 h and 4 h adaptations, respectively. Fitting the experimental data with three or more distinct mutations requires very large mutational probability $\sim 10^{-3}$. This high probability seems unreasonable due to the expected mutation rate in this strain

on the order of 10^{-9}/bp/generation, although it is in principle possible that a higher mutation rate for this process occurs for unknown reasons.

In summary, the simulation indicates that two mutations are needed to explain the data, and that the mutations with the larger effect on lag phase become fixed first (it is not possible to conclude from the present data whether the effect of the second mutation depends on the first mutation).

TWO DISTINCT MUTATIONS THAT REDUCE LAG PHASE ARE EXPERIMENTALLY FOUND

Based on these simulation predictions, we experimentally searched for the two distinct predicted mutations. The calculations predict that the population in the first generations of the experiment should mainly consist of three cell types: the wild type, mutants with a 6 h reduction of the lag phase and mutants with a 10 h reduction of the lag phase (that is, bacteria that carry both 6 h and 4 h mutations). According to the simulation, mutants with only a 4 h reduction of the lag phase should be rare because they cannot compete with the 6 h mutant. The 4 h mutation can be observed only as a second mutation that occurred in a strain with the 6 h mutation.

To test this we isolated 96 colonies from the experimental cell population after 35 generations of evolution, and measured the duration of their lag phases (Fig. 3). The isolates consisted mostly of mutants that have a 6 h lag-phase reduction. Around 15% of the isolates showed a 10 h reduction of the lag phase and 10% of the isolates have the wild-type lag period of 15 h. These results are in reasonable agreement with the predicted phenotype frequencies (Fig. 3).

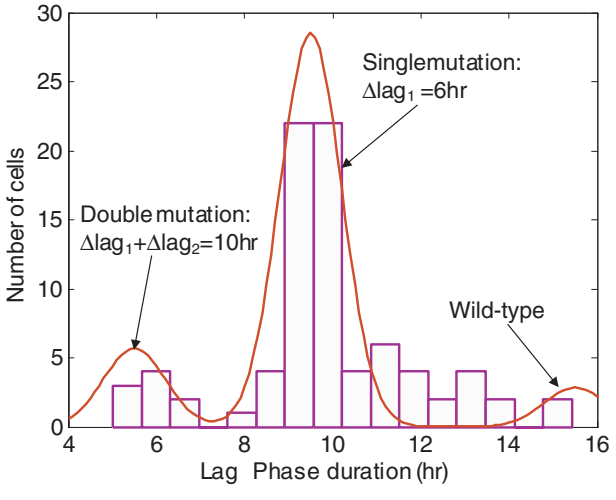

Figure 3. Distribution of the lag-phase duration of 96 colonies isolated from the population after 35 generations of evolution. The bars are the experimental measurements, and the solid line is the simulation results. The solid line describes the calculated frequency of the wild type, a mutant with a single mutation that shortens its lag period by 6 h and a mutant with two mutations that shortens its lag period by 10 h, with an additional Gaussian measurement error with an standard deviation of 1 h.

SIMULATION AND EXPERIMENT SUGGESTS THAT GROWTH RATE ADAPTATION OCCURS BY TWO DISTINCT MUTATIONS

We now turn to analyze the second stage of adaptation (above 100 generations). This stage begins with a population consisting of cells with a reduced lag phase. The next observable adaptation increases the exponential growth rate. This adaptation is noticeable after 150 generations, and dominates the population after another 150 generations.

As in the adaptation of the lag phase, comparison of the simulation to the experiments suggests that the cumulative effects of two different mutations are required to explain the dynamics of adaptation in the second stage. The first mutation increases growth by $(20 \pm 5)\%$ and the second by $(15 \pm 3)\%$. The predicted mutational probabilities for these adaptations are both $P = (1 \pm 0.5) \times 10^{-8}$.

THE ORDER OF ADAPTATIONS IS REVERSED IN MEDIA SUPPLEMENTED WITH LACTOSE

Finally, we asked whether the adaptation order could be reversed. Can one find an environment in which the growth rate adaptation will be observed before the lag-phase adaptation? To address

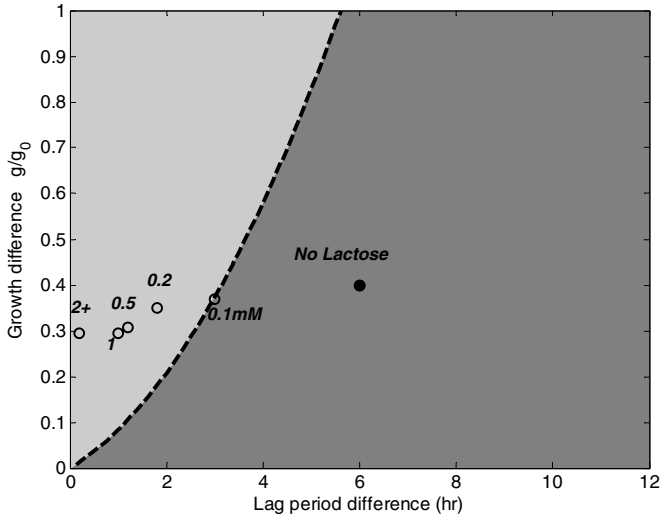

Figure 4. Adaptation phase space. Each point in this phase space indicates the mutant lag-phase reduction (x-axis) and relative growth rate increase (y-axis). The dark gray area indicates mutants in which the lag-phase adaptation occurs before the growth rate adaptation. The light gray area indicates the opposite adaptation order. Circles indicate the present experimental data, in which serial dilution experiments were performed in succinate minimal medium with different levels of lactose. The full circle is for evolution with no lactose.

this question we used population dynamics theory to calculate an "adaptation phase-space" shown in Figure 4 (see Methods for an analytical derivation of this phase space).

The adaptation phase space describes the order of fixation of pairs of adaptations: the x-axis is the reduction in lag duration by one adaptation, and the y-axis is the increase in growth rate by the other adaptation. Points located on the right side (dark gray area) of the phase space show an order of fixation where the lag adaptation becomes fixed first, followed by the growth rate adaptation. Points in the light area are predicted to show the opposite order, growth and then lag.

The experiment described above (Fig. 2) is indicated by the full circle and is located in the area where the 6 h lag-phase mutants are the first to be fixed, before the growth rate mutants. To experimentally examine the left side of the adaptation phase space, where the growth rate adaptation is expected to occur first, we supplemented the medium with the sugar lactose. Lactose reduces the duration of the lag phase of the bacteria. The higher the lactose concentration, the shorter the lag duration. We conducted seven evolutionary experiments in parallel, which differ in the lactose concentration: 0.1 mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM, 5 mM, and 10 mM. Serial transfer in each of these conditions was performed for about 500 generations, using the protocol described above. The lag duration of the wild-type bacteria in these lactose concentrations was measured to be 5.5 h, 4 h, 3.5 h, 3 h, 2 h, 2 h, and 2 h, respectively, and the exponential growth rate was measured to be 0.46 h^{-1}, 0.52 h^{-1}, 0.62 h^{-1}, 0.69 h^{-1}, 0.69 h^{-1}, 0.69 h^{-1}, and 0.69 h^{-1}, respectively.

We analyzed the course of evolution for all of seven serial dilution experiments. The measured final adaptations of the lag period and growth rate are indicated by the open circles in Figure 4. In all of these conditions, we find that the growth rate adaptation occurs before the lag-phase adaptation, as predicted by the theory. Hence, the order of adaptations is reversed compared to growth on the same medium not supplemented by the sugar lactose.

Figure 5 shows the course of evolution in the experiment with 0.2-mM lactose. Adaptation of the growth rate was complete after less than 100 generations (Fig. 5B,C) whereas adaptation of the lag phase was seen only after 150 generations (Fig. 5A,C). The simulation results for this experiment are indicated by the blue curve in the figure. The simulations suggest that in this condition, it took a single mutation to adapt the growth rate (with $P = (5 \pm 2) \times 10^{-7}$), and two mutations to adapt the lag phase (with $P = (4 \pm 2) \times 10^{-6}$, $(1 \pm 0.5) \times 10^{-8}$).

DISCUSSION

This study presented a case in which the evolutionary path to adaptation is almost deterministic, and can be understood in terms

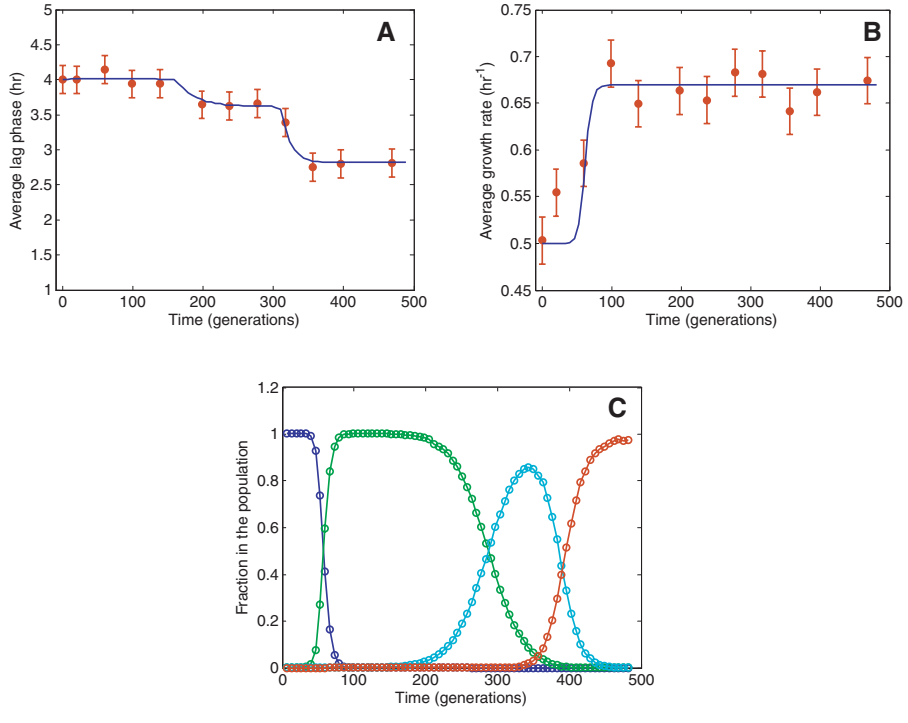

Figure 5. Evolution in which order of adaptations is reversed, in succinate medium supplemented by 0.2 mM lactose. (A) Lag-phase duration as a function of the number of generations, experiment (circles), and calculation (solid line). (B) growth rate experiment (circles) and calculation (line). (C) Calculation of the relative populations of wild type (blue line) and mutants as a function of evolutionary time. The first mutation is of growth rate (green line) followed by two lag-phase mutations (reducing lag period by 0.4 $\pm$ 0.1 h, cyan, and 0.8 $\pm$ 0.2 h, red).

of theoretical analysis. In media where the lag phase is long, adaptations that reduce lag phase have a highly beneficial effect. This is because mutants with a shorter lag phase start their exponential growth earlier and can consume most of the environmental nutrients before the wild type exit the lag phase. Therefore, in these conditions, lag-phase adaptations are fixed before adaptations that affect the growth rate. In conditions in which lag phase is short, growth rate adaptations can become fixed before lag-phase adaptations. Population genetics theory was useful in pointing out the existence of cumulative events in achieving each adaptation, and in predicting the conditions under which the order of adaptations is reversed.

In the wild, as part of its life cycle, *E. coli* is thought to periodically enter and exit from stationary phase, where it spends a considerable time (Cooke 1974). Therefore, one might expect a trade-off, where it may be advantageous to shorten the lag phase to some degree at the expense of reducing the probability of survival in stationary phase (see online Supplementary Fig. S3).

An interesting question is whether the different adaptations have effects that are dependent on each other or are independent. The theory suggests that in large enough populations, adaptations with a large effect should be fixed before adaptations of smaller effect, provided that the former is not dependent on the latter (Fisher 1930). When adaptations with a smaller effect are fixed before adaptations with a larger effect, one can hypothesize that the effects of the second adaptation can only be realized in a background containing the first adaptation. Such a case can be seen in the present experiments in Figure 5A, where an adaptation that reduces lag phase by 0.5 h is fixed before a second adaptation that effects lag phase by a further 1 h.

To a first approximation it seems that lag phase and exponential growth rate are separable in terms of beneficial mutations, and thus that there is no pleiotropy. This is only approximately true, as evident from the finding of a mutation that had a considerable affect on the lag phase but also a mild affect on growth (Fig. 2). This effect suggests that lag and growth phases are coupled, and in some cases cannot be considered fully separable.

There is a considerable difference between phenotypic and genetic determinism. The data support phenotypic determinism in the present case. They do not imply genetic determinism. For example, a previous study on the evolution of lac protein expression showed that adaptation of a population occurred by multiple co-existing mutants that had very similar phenotype but different genotypes (Dekel and Alon 2005).

The present study focused on the question of the uniqueness of the path to adaptation. Future work can attempt to identify the precise substitutions in the DNA underlying these adaptations, for example by genome resequencing (Herring et al. 2006). It would be interesting to extend this study to other conditions in which adaptation occurs in multiple steps, such as pathways in which both regulators and enzymes contribute to the overall function (Crameri et al. 1997; Segre et al. 2006). The present approach can be readily extended also to study evolutionary paths in other micro-organisms (Segre et al. 2006).

Methods

STRAINS AND MEDIA

Escherichia coli MG1655 (*E. coli* genetic stock center) was used. All experiments were in M9 defined medium consisting of M9 salts, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1 mM IPTG (for lac expression to compare between mediums with and without lactose) and 0.25% succinate as a carbon source. Some of the experiments contained a specified concentration of D-lactose (Sigma). The growth conditions of *E. coli* in the used concentration of succinate are very poor. The lag-phase period is about 15 h and the growth rate is about 0.42 h^{-1}.

GROWTH RATE MEASUREMENTS

The exponential growth rate difference of two strains was measured by diluting the bacteria 1:100 from overnight culture, started from frozen stocks, and growing them in a multiwell fluorimeter (Wallac Victor 2) with shaking at 37°C, with OD measurements every 12 min over 26 h of growth. Cells were grown in wells containing 200 μl of medium covered by 50 μl mineral oil (Sigma) to avoid evaporation. Online Supplementary Figure S4 compares the growth of bacteria in 50 mL shaken tubes and growth in 200 μl multi-well plate, and shows that exponential growth rate in the multi-well plate is equal to within experimental resolution to that in the 50 mL tubes.

DETERMINATION OF THE LAG TIME

We determined the lag time by extrapolating the tangent at the exponential part of the growth curve, back to the initial size of the population. The lag time was set to the intersection point (Pirt 1975; Zwietering et al. 1992).

SERIAL DILUTION EXPERIMENTS

Ten milliliters cultures were grown in 50 mL tubes shaken at 220 rpm at 37°C. After two days of growth, cells were diluted 1:100 into a fresh tube. Samples were frozen (−80°C) every three days (Cooper et al. 2003).

SIMULATIONS OF POPULATION DYNAMICS

Population dynamics simulation was performed as follows (Hartl and Clark 1997): A population of wild-type cells starts to grow exponentially at a growth rate g_0 after a lag period l_0. Mutants are formed with a probability p_k per generation per cell. There are two kinds of mutants: lag-phase mutants having shorter lag-phase

period $l_0 - \Delta l$, and growth rate mutants having a faster growth rate $g_0+\Delta g$. The parameters g_0, l_0, Δg, and Δl were set equal to the measured growth rate and lag phase of the wild-type and mutant strains. At the end of each simulated day, 1/100 of the population was randomly transferred to the next simulated day, of which the fraction of mutants was determined by a random binomial process. The growth rate and lag phase of the population was calculated as the average of the population. The resulting dynamics shows the hierarchy of mutants that eventually take over the population. The simulations have free parameters, p_k, which is the probability for the kth mutation per cell per division that we fitted to the experimental data. The order of magnitude of p_k was fitted to 10^{-8}–10^{-6}, which corresponds to a target size of 10–1000 (assuming that the wild-type mutational rate is 10^{-9}).

ANALYTICAL DERIVATION OF THE ADAPTATION PHASE SPACE (FIG. 4)

The adaptation phase space indicates the order of adaptations. Mutants located in the dark gray area will acquire the lag-phase adaptation before the growth rate adaptation and opposite order for mutants located in the light gray area. On the boundary between the dark and light area the benefit from the lag and growth adaptation is equal. We can therefore write that on this boundary the number of mutants that increased their growth rate by Δg will be equal to the number of mutants that reduced their lag period by Δl:

$$N_0 e^{(g_0+\Delta g)(t-l_0)} = N_0 e^{g_0(t-l_0+\Delta l)}$$

where g_0, l_0 are the initial growth rate and lag period, respectively, N_0 is the initial number of bacteria and t is time. Equating the exponents, we get: $\Delta\, g(t - l_0) = g_0\, \Delta\, l$.

We set the time t to the serial dilution time at the end of the day:

$$t - l_0 = \frac{\ln\left(\frac{N_f}{N_0}\right)}{g_0 + \Delta g},$$

where N_f is the final number of bacteria before the serial transfer. Substituting $t - l_0$, we get:

$$\frac{\Delta g}{g_0 + \Delta g} \ln\left(\frac{N_f}{N_0}\right) = g_0 \Delta l$$

solving for Δg, we finally get

$$\frac{\Delta g}{g_0} = \frac{g_0 \Delta l}{\ln\left(\frac{N_f}{N_0}\right) - g_0 \Delta l}$$

in the evolutional experiment, we measured $g_0 = 0.42\ \mathrm{h}^{-1}$, and $N_f/N_0 = 100$.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. Distribution of the lag phase duration of 96 colonies isolated from the wild-type population.

Figure S2. Comparison of growth between wild-type and mutant strain (evolved in M9+succinate for 100 generations) in a medium of M9+glycerol, which is different than the medium used for evolution.

Figure S3. Comparison of the survival during stationary phase between evolved and wild-type strains.

Figure S4. Growth rate of *E. coli* in M9C medium in 200 μl wells measured by OD_{600} in multiwell photometer.

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