

Evolution of competitive fitness in experimental populations of *E. coli*: What makes one genotype a better competitor than another?

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Abstract

An important problem in microbial ecology is to identify those phenotypic attributes that are responsible for competitive fitness in a particular environment. Thousands of papers have been published on the physiology, biochemistry, and molecular genetics of *Escherichia coli* and other bacterial models. Nonetheless, little is known about what makes one genotype a better competitor than another even in such well studied systems. Here, we review experiments to identify the phenotypic bases of improved competitive fitness in twelve *E. coli* populations that evolved for thousands of generations in a defined environment, in which glucose was the limiting substrate. After 10000 generations, the average fitness of the derived genotypes had increased by ~ 50% relative to the ancestor, based on competition experiments using marked strains in the same environment. The growth kinetics of the ancestral and derived genotypes showed that the latter have a shorter lag phase upon transfer into fresh medium and a higher maximum growth rate. Competition experiments were also performed in environments where other substrates were substituted for glucose. The derived genotypes are generally more fit in competition for those substrates that use the same mechanism of transport as glucose, which suggests that enhanced transport was an important target of natural selection in the evolutionary environment. All of the derived genotypes produce much larger cells than does the ancestor, even when both types are forced to grow at the same rate. Some, but not all, of the derived genotypes also have greatly elevated mutation rates. Efforts are now underway to identify the genetic changes that underlie those phenotypic changes, especially substrate specificity and elevated mutation rate, for which there are good candidate loci. Identification and subsequent manipulation of these genes may provide new insights into the reproducibility of adaptive evolution, the importance of co-adapted gene complexes, and the extent to which distinct phenotypes (e.g., substrate specificity and cell size) are affected by the same mutations.

Introduction

What phenotypic properties make one bacterial genotype a superior competitor to another in a certain environment? Unfortunately, we cannot provide any general answers to this question at the present time. Nonetheless, we believe that this question is sufficiently important to the field of microbial ecology that it is worthwhile to consider how one might go about getting an answer, even if only in some special circumstances. To that end, we will summarize our progress to date in

understanding the phenotypic – and ultimately genetic – bases of enhanced competitive fitness in a model experimental system. This review focuses on one aspect of a long-term evolution experiment that is ongoing in our laboratory. Other reviews on experimental studies of microbial evolution can be found in papers by Dykhuizen & Hartl (1983), Hall (1983), Levin & Lenski (1983), Mortlock (1984), Dykhuizen & Dean (1990), and Lenski (1992, 1995).

Before we describe our experimental system, let us consider the general nature and scope of the problem.

Of course, it would be highly desirable to be able to predict the outcome of competition between bacterial genotypes, especially in such managed ecosystems as bioreactors and agricultural fields. Yet who among us can do so? Many thousands of person-years have been devoted to investigations of the physiology, biochemistry, and molecular genetics of one bacterial species, *Escherichia coli* (Neidhardt et al. 1996). But ask even the most knowledgeable student of *E. coli* to predict, with any accuracy, which of two genotypes would prevail in competition in a particular environment. Aside from trivial cases – for example, that a Lac⁺ strain should displace a Lac[−] mutant when lactose is the sole source of carbon and energy – most microbiologists would readily admit that they could not do so.

Why not? Most microbiologists tend to ‘atomize’ an organism, studying one gene or pathway at a time, rather than viewing the organism as an integrated whole. Microbiologists also usually pursue the traditional genetic approach of determining structures and their functions by using defective mutants, whereas many of the important differences between genotypes that would influence the outcome of their competition may involve subtle quantitative variation. Additional complications arise because any two laboratory-derived genotypes may differ by mutations at several loci, and two natural isolates of the same ‘species’ may differ at the level of their nucleotide sequences by several percent (hence, tens of thousands of base-pair differences). All of these issues pertain to the complexity of the organisms, and many additional challenges arise from the complexity and unpredictability of their environments. Perhaps, then, we should consider other approaches to studying the determinants of competitive fitness in bacteria – even if there are no guarantees that an alternative approach will succeed. In this paper, we examine one alternative approach, in which populations of bacteria are allowed to evolve in a simple laboratory environment and the resulting phenotypic and genetic changes are then analyzed.

Overview of the experimental system

Twelve populations of *E. coli* B were founded from a single ancestral strain, and these populations have now been propagated for more than 10000 generations in a relatively simple, defined environment (Lenski et al., 1991; Lenski & Travisano, 1994). The primary motivation for this experiment was to address basic questions about the dynamics of evolutionary adapta-

tion and divergence. Could one observe the process of adaptation by natural selection, whereby the performance of the bacteria in the experimental environment would tend to improve over time? Would the rate of their improvement be essentially constant over time, or would the populations show episodes of faster and slower evolutionary change even in a constant environment? Would all twelve replicate populations improve their performance to a similar extent, or would some of the populations find better solutions to the environment than would others?

During the course of this evolution experiment, we became increasingly interested in the phenotypic and genetic changes that made the derived bacteria better adapted to the experimental environment than were their ancestors. A mechanistic understanding of these changes would allow us to answer with greater precision some of our basic questions about evolutionary dynamics. But we also saw a unique opportunity to address the question of what makes one genotype a better competitor than another. On the one hand, the changes that occur in the evolving populations are more numerous and more subtle in their effects than the ‘one-at-a-time knockout’ mutations that are typically studied by microbial geneticists. And on the other hand, these evolving populations and their environment are far simpler than the natural systems that are typically studied by microbial ecologists.

The ancestral genotype in the evolution experiment was prototrophic, but it also had several genetic markers that allowed us to exclude the possibility of external contamination (Lenski et al., 1991). We also had another marker that was embedded in the experimental design itself, which allowed us to exclude cross-contamination among the replicate populations. This same marker allowed us to put evolutionarily derived genotypes in competition with the ancestral strain of the opposite marker state and thereby directly measure the changes in competitive fitness. Prior to experiments to measure competitive fitness or any other property, both derived and ancestral genotypes were removed from storage at −80 °C and acclimated to the experimental environment. Thus, both ancestral and derived genotypes were in the same physiological state, and any significant differences between them must have a genetic basis.

The twelve evolving populations were serially propagated at 37 °C in Davis minimal medium supplemented with 25 µg of glucose per mL. Each day, 0.1 mL of the previous day’s culture was transferred into 9.9 mL of fresh medium. The 100-fold dilution

and regrowth allowed $\sim 6.6 (= \log_2 100)$ generations of binary fission each day, and the 10000 generations of experimental evolution required 1500 daily transfers. Each day, the evolving cell populations experienced lag, exponential growth, decelerating growth, and stationary phases as the total size of each population fluctuated between $\sim 5 \times 10^6$ and $\sim 5 \times 10^8$ cells per 10 mL culture.

The ancestral strain in our experiment did not carry any transmissible plasmids or viruses; thus, its evolution was strictly clonal (asexual). Mutations that occurred during the evolution experiment therefore provided all of the genetic variation that was available to these populations. However, the mutational input was actually quite large. Given $\sim 5 \times 10^8$ cell replications per day for 1500 days, and an estimated genomic rate of 0.0025 mutations per cell replication (Drake, 1991), there must have been at least 10^9 mutations in each of the evolving populations (Lenski & Travisano, 1994). By contrast, the genome size of *E. coli* is only $\sim 5 \times 10^6$ basepairs, and so there were more than 100 mutations, on average, at each site in the genome. These calculations may actually underestimate the number of mutations to the extent that certain loci are hypermutable and transposition events may not always be counted. Of course, most of these mutations would have been lost during each serial transfer and not all sites are equally mutable. Even so, mutation clearly provided a tremendous amount of genetic variation, which would allow the process of adaptation by natural selection to proceed.

Experimental findings

Changes in competitive fitness

Figure 1 shows the trajectory for the grand mean fitness (averaged over the 12 replicate populations) during 10000 generations of experimental evolution with *E. coli*. Fitness values were measured by placing derived populations in competition with the ancestral strain (bearing the opposite genetic marker) in the same environment used for the experimental evolution. Fitness is expressed relative to the common ancestor, and it is calculated simply as the ratio of the growth rates realized by the two competitors during their head-to-head competition.

After 10000 generations, the derived genotypes are $\sim 50\%$ more fit than their ancestors, with about half of this improvement occurring in the first 2000 gener-

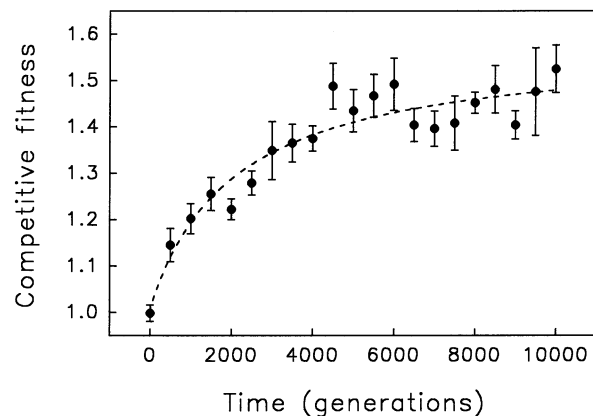


Figure 1. Change in competitive fitness during 10000 generations of experimental evolution with *E. coli*. Fitness is expressed relative to the common ancestor. Each point is the grand mean averaged over twelve replicate populations. Error bars are 95% confidence intervals. The dashed curve indicates the best fit of a hyperbolic model to the data. Data from Lenski & Travisano (1994).

ations. Based on more detailed analyses of the kinetics of mutation and selection, we estimate that only a handful of mutations – about four, on average – can account for the improvement seen during these first 2000 generations (Lenski et al., 1991; Lenski & Travisano, 1994; Elena et al., 1996; Gerrish & Lenski, 1998). Perhaps ten or so mutations can account for all of the improvement in any population over the entire 10000 generations. In addition to these beneficial mutations, other mutations may have drifted to high frequency in a population by random sampling during the daily transfers or by hitchhiking along with a beneficial mutation that happened to occur in the same cell. Using the theoretical expectation that the steady-state substitution rate for selectively neutral alleles is equal to their mutation rate (Kimura 1983), then perhaps another twenty to thirty mutations may have been fixed in each population by random drift. This calculation is based on the total genomic mutation rate for *E. coli* (~ 0.0025 mutations per genome replication: Drake, 1991), and it further assumes that a substantial fraction of all mutations are in fact essentially neutral. In other words, despite the tremendous number of mutations that occurred in each of these populations, only a relatively few are likely to have increased to a high frequency.

How can we determine the mechanistic bases by which the derived bacteria have become better adapted to their environment? We will briefly consider the advantages and disadvantages of three approaches that are widely used in molecular biology, genetics, and

evolutionary biology before describing the approach that we have taken.

Some alternative approaches to characterizing the changes responsible for improved competitive fitness

A seemingly straight-forward molecular approach would be to obtain and compare DNA sequences from the ancestral and derived genotypes. Sequencing entire genomes, however, is still a formidable problem. Unless the specific genes in which the changes have occurred can somehow be identified, one must use methods, such as RFLP analysis, that can detect changes in a sample of the basepairs around the genome. Although these methods are now relatively simple and inexpensive, the fraction of the genome that can be sampled is still quite small. For example, a restriction enzyme that recognizes a particular 8-bp sequence can be expected to detect, on average, only about one out of every 10,000 point mutations in the *E. coli* genome. (One would expect to detect a higher fraction of insertion and deletion mutations by this approach.) But we estimated that there are probably no more than about forty genetic differences between the ancestral and derived genotypes, and so a large battery of restriction enzymes would be needed to detect any changes that were due to point mutations. Moreover, any genetic changes that were detected using this approach might have occurred by random drift or by hitchhiking with a beneficial mutation elsewhere in the genome. Thus, having found a difference in DNA sequence between derived and ancestral genotypes, there is no guarantee that it is causally related to the difference in their relative fitness. Thus, while 10000 generations is a very long time for an experiment, it is actually a very short time from the standpoint of molecular evolution, and molecular changes do not necessarily imply important phenotypic changes.

Another approach would be to use the classical bacterial genetic methods of conjugation, transduction, and transformation to map the genes that enhance fitness. However, there are severe technical difficulties with implementing this approach. Chief among them is the fact that these classical approaches work well when there are qualitative differences in phenotype, such that a genotype either can or cannot grow on a particular medium. In that case, one can cleanly counter-select against both parental strains and thereby find the recombinant 'needle in a haystack.' But the phenotypic differences that interest us are subtle changes in competitive fitness. One might circumvent this problem by

using parental strains that differed at a large number of marker loci that could be readily counter-selected, and then perform a linkage analysis to find markers associated with fitness effects (in essence QTL mapping applied to bacteria). However, fitness as a trait may be influenced by so many genes that it seems likely that the marker loci themselves would have individual or epistatic effects on fitness, thereby confounding the analysis and its interpretation. Thus, the classical approach to mapping bacterial genes is well suited to the 'plus or minus' phenotypes used in studies that seek to identify structure and function by knocking out an underlying gene; but this approach is not so useful for mapping genetic changes that underlie the more subtle phenotypic refinements that are often important during evolution.

Evolutionary biologists who study the effects of natural selection in contemporary populations of animals and plants often employ a very different approach. In essence, this approach begins with the observation that certain phenotypic traits appear to be especially important for the ecological performance of a population or species in the wild. For example, observations on one of Darwin's finches, *Geospiza fortis*, suggested that beak size and shape might affect the birds' success in foraging on several different kinds of seeds, which became more or less abundant depending on patterns of rainfall (Price et al., 1984). To build a rigorous case that natural selection is indeed changing these traits in an adaptive manner, one then faces a two-fold challenge. First, one must show that variation in the traits is at least partly heritable, so that offspring tend to resemble their parents owing to genetic similarity (as opposed to shared environmental influences). Second, one must show that differences in the traits are significantly associated with differences in the survival and reproductive success of individual organisms. In essence, it is necessary to fulfill these conditions in order to infer heritable differences in fitness. Not surprisingly, such analyses are very difficult in most systems given the lack of experimental control over both genetic factors and environmental variables.

By contrast, it has been relatively simple to demonstrate heritable differences in fitness between ancestral and derived genotypes in our experimental populations of *E. coli*. In the following sections, we present the results to date of a multifaceted 'top-down' analysis that seeks, first, to identify heritable phenotypic differences between the ancestral and derived genotypes, and then to explain the mechanistic basis for the enhanced competitive fitness of the derived bacte-

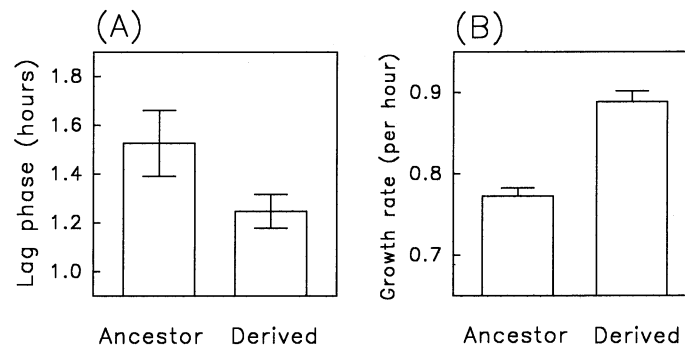


Figure 2. (A) The derived genotypes have a significantly shorter lag phase, upon transfer to fresh medium, than does the ancestral strain. (B) The derived genotypes have a significantly faster maximum growth rate at high glucose concentration than does the ancestral strain. Error bars are 95% confidence intervals. Data from Vasi et al. (1994).

ria. As in all evolutionary studies, merely showing a correlation between some phenotypic trait and fitness will not be sufficient to prove that changes in that trait were responsible for the fitness gains in the derived genotypes. However, as putative phenotypic targets of selection are identified, we hope eventually to incorporate classical and molecular approaches to identify and manipulate the genetic determinants of enhanced competitive fitness.

Changes in demographic parameters

Our first level of analysis sought to determine at what stages in the population growth cycle the derived bacteria gained an advantage relative to their progenitors (Vasi et al., 1994). Every day during the long-term evolution experiment, the bacteria were diluted into fresh medium, where they exhausted the available glucose before the next transfer. This cycle of 'feast and famine' was repeated during the experiments used to quantify competitive fitness. In principle, the derived bacteria could have become better adapted to this regime by shortening the duration of their lag phase prior to growth, increasing their maximum growth rate, improving their growth rate at low glucose concentrations (i.e., reducing K_s), or by reducing their death rate after the glucose was exhausted.

We estimated all of these growth parameters for the common ancestor and for derived genotypes sampled from each of the populations after 2000 generations. As shown in Figure 2, the derived genotypes had significantly shorter lag phases and significantly higher maximum growth rates (U_{max}) than their ancestor. However, the derived genotypes showed no significant improvement relative to their ancestor in either the

concentration of glucose required to support growth at half the maximum rate (K_s) or in their death rates during stationary phase. In fact, the derived genotypes had significantly higher values for K_s than did their ancestor, although the ratio of U_{max}/K_s (which determines the rate of growth as the glucose concentration approaches zero) had not changed significantly. Evidently, the derived bacteria had become better adapted to exploiting the periods of 'feast' that occurred every day, but they were not any better (or worse) than their ancestor in terms of tolerating the periods of 'famine.' The failure of the derived bacteria to become better adapted to these periods of famine probably reflects the fact that the ancestral genotype was already so well adapted to these conditions. That is, the ancestral strain experienced little or no death during the 14–16 hours after glucose was exhausted (prior to the next transfer into fresh medium), and it could grow at greater than half its maximum rate even after more than 99% of the daily supply of glucose was depleted.

Although the derived genotypes increased in abundance *relative* to the ancestral genotype during competition experiments, we also observed that the numerical yield (cfu per mL) of the derived genotypes was lower than that of the ancestor when they were grown in isolation. In principle, the reduced numerical yield of the derived bacteria could reflect several different changes: (1) the derived bacteria might have lower plating efficiency than the ancestor; (2) the derived bacteria might grow faster, but less efficiently, on glucose than the ancestor; and (3) the derived bacteria might have cells that are larger in size but fewer in number than the ancestor.

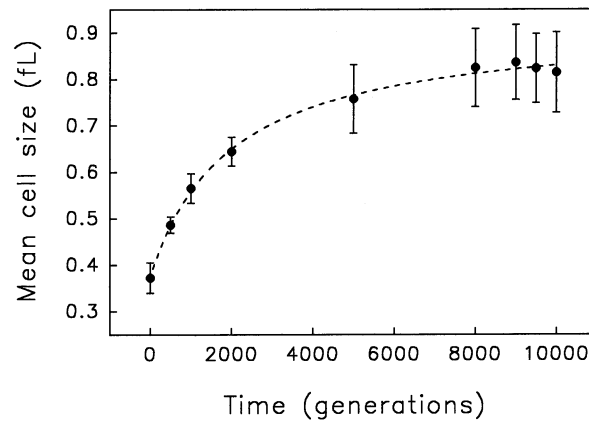


Figure 3. Change in average cell size during 10000 generations of experimental evolution with *E. coli*. Cell size is expressed volumetrically, where 1 fL = 10^{-15} L. Each point is the grand mean averaged over twelve replicate populations. Error bars are 95% confidence intervals. The dashed curve indicates the best fit of a hyperbolic model to the data. Data from Lenski & Travisano (1994).

Changes in cell size

We used a Coulter particle counter to measure the volume displaced by individual cells of the derived and ancestral genotypes (Vasi et al., 1994; Lenski & Travisano, 1994). Derived genotypes from all twelve evolving populations produced significantly larger cells than did their ancestor. Figure 3 shows the evolutionary trajectory for the grand mean cell volume. After 10000 generations, the derived genotypes produced cells that were, on average, approximately twice as large as those produced by their ancestor. Thus, the evolved populations have cells that are larger in size, but fewer in number, than the ancestral population. However, the increase in average cell size and the reduction in cell number do not exactly balance one another. Rather, there was a net increase in the biovolume yield of the derived populations (Vasi et al., 1994), indicating that the derived genotypes have become more efficient than their ancestors during growth on glucose.

One might have expected that *smaller* cells would have a selective advantage by virtue of their larger ratio of surface area to cell volume, which would facilitate nutrient transport. Of course, this ratio depends on the shape as well as the size of cells. Microscopic examination shows that the derived genotypes significantly increased both the length and the diameter of their cells relative to the ancestor (J.A.M. & R.E.L., unpubl.). But it is noteworthy that the derived cells typically increased their length to a somewhat greater extent than they increased their width. Thus, while the ratio of surface area to volume declined substantially relative to the ancestor, it did so to a lesser degree than

if the cells had increased in size isometrically (i.e., without changing shape).

It is unclear what advantage might accrue to larger cells in the experimental environment. Perhaps larger cells have greater metabolic reserves, so that they can respond more quickly to the sudden increase in nutrient availability that occurs at each daily transfer. This explanation is consistent with the reduced length of the lag phase in the derived genotypes. However, there is another explanation that does not suppose that increased cell size *per se* was the actual target of natural selection. Bacterial physiologists have shown that there is a positive correlation between cell size and growth rate when bacteria are grown on different media (Schaechter et al., 1958; Bremer & Dennis, 1987; Akerlund et al., 1995). This effect is strictly phenotypic and does not involve any genetic change. By extension, it is possible that the larger cell size of our derived genotypes reflects the simple fact that they grow faster than does their ancestor. In other words, the larger cell size of the derived genotypes might simply be a correlated response to selection for faster growth, rather than reflecting any direct advantage to larger cells (Figure 4A).

In fact, this hypothesized explanation for the evolution of larger cell size can be readily tested by forcing the derived and ancestral genotypes to grow at the same rate by culturing them in separate chemostat vessels (Mongold & Lenski, 1996). When we did so, we observed the previously reported relationship between cell size and growth rate for both derived and ancestral genotypes; that is, faster growing cells were also phenotypically larger. However, the derived genotype

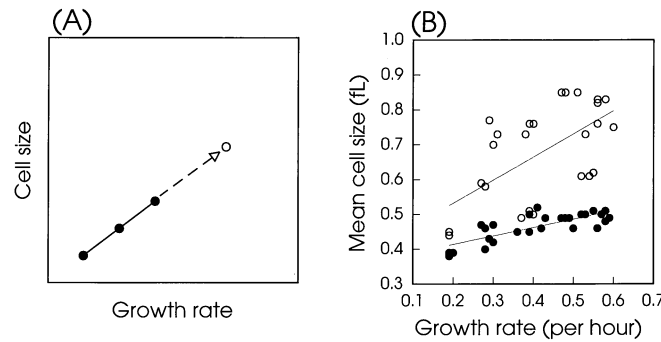


Figure 4. (A) Hypothetical non-adaptive mechanism for an evolutionary change in cell size due to a phenotypic correlation with growth rate. Filled circles: cell size of the ancestral genotype is phenotypically correlated with its growth rate. Open circle: a mutation that allowed a derived genotype to grow faster would cause a correlated increase in cell size, even though the mutation was in a gene that did not directly control cell size. (B) This hypothetical non-adaptive mechanism is rejected by experiments in which ancestral and derived genotypes are forced to grow at the same rate in independent chemostat vessels. Filled circles: relationship between mean cell size and growth rate in the ancestral genotype. Open circles: relationship between mean cell size and growth rate in a derived genotype obtained after 2000 generations of experimental evolution. The derived genotype produces larger cells than the ancestor even when they are grown at the same rate. Panel B reprinted with permission from Mongold & Lenski (1996).

was significantly larger than its ancestor when the two strains were growing at the same rate, except at very low growth rates (Figure 4B). Thus, the phenotypic correlation between cell size and growth rate cannot fully account for the evolutionary change in cell size that we observed, and some other explanation must be sought.

Changes in cell composition

We have also begun experiments to examine whether the macromolecular composition of the evolving cells has changed and, if so, how this scales with cell size (J.A.M., unpubl.). The derived genotype has more protein, RNA, and DNA per cell than does its ancestor, which is not surprising given that the derived cells are generally larger. When the amounts of protein, RNA, and DNA are expressed per unit of cell volume, the concentrations of protein and RNA are indistinguishable between the derived and ancestral genotypes, whereas the concentration of DNA is actually somewhat lower in the derived genotype. Thus, the ratio of protein to RNA has remained constant, while the ratio of RNA to DNA appears to have increased in the derived genotype. Of course, most of the RNA in a cell is ribosomal.

Comparative data indicate the copy number of ribosomal (*rrn*) operons is evolutionarily labile; while *E. coli* typically has seven *rrn* operons, other bacterial species have between 1 and 14 *rrn* operons (Schmidt, 1996). Given the increased ratio of RNA to DNA in the derived genotype, we wanted to see whether the

rrn copy number might have increased in the evolving populations (T.M.S., unpubl.). To that end, we isolated genomic DNA from the reciprocally marked ancestral genotypes and from derived genotypes sampled from all twelve populations after 10000 generations. This DNA was cut with restriction enzyme *PvuII* and then probed with a DIG-labeled transcript from positions 874 to 1382 of the 16S RNA-encoding gene of the *E. coli rrnB* operon. This enzyme cuts a unique site in each *rrn* operon, and that site is located such that even a tandem duplication of an *rrn* operon should yield an additional fragment that could be detected using that probe. As shown in Figure 5, all twelve independently derived genotypes retain the seven ancestral *rrn* operons, without any additional copies being present. Thus, we can reject an increase in the number of ribosomal operons as the explanation for the increased ratio of RNA to DNA in the derived genotype.

It will also be interesting to obtain information on other aspects of cellular composition, including cell envelope proteins as well as total carbohydrate and lipid content. An increase in the stationary-phase levels of storage molecules would corroborate the hypothesis that larger cells have greater metabolic reserves, such that they can respond more quickly to the daily influx of nutrients. Also, the stoichiometry of the envelope might be such that the amounts of key proteins involved in glucose transport (see below) cannot be raised without simultaneously increasing the surface area of the cell. If so, this could provide an alternative explanation for the evolutionary trend to larger cell size.

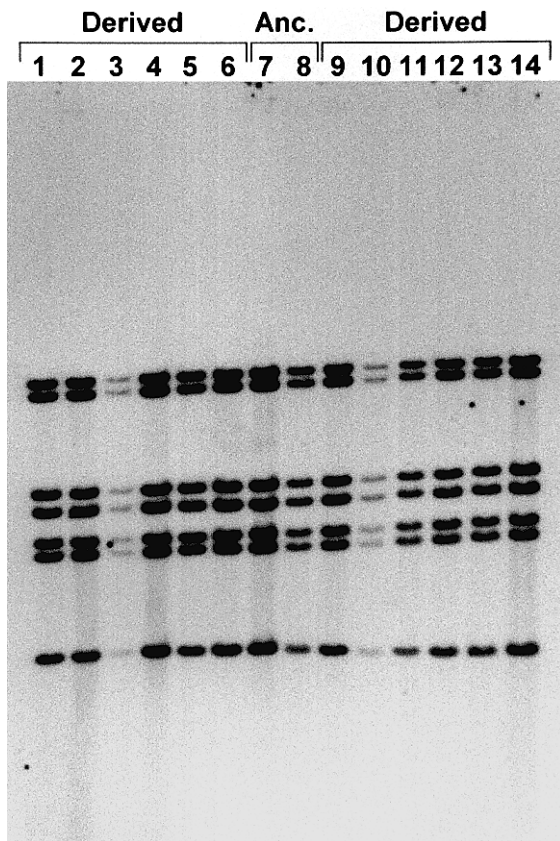


Figure 5. Ancestral and evolutionarily derived genotypes of *E. coli* have the same number of *rrn* operons. *PvuII*-restricted genomic DNA was hybridized with a labeled fragment of *E. coli* 16S RNA. The resulting Southern blot indicates the constancy of *rrn* copy number as well as the position of *PvuII* restriction sites adjacent to the *rrn* operons. Shown from left to right are: genotypes from six of the evolving populations after 10000 generations; the two reciprocally marked ancestral genotypes; and genotypes from the other six evolving populations after 10000 generations. Unpublished data of T.M.S.

In summary, the evolving populations underwent dramatic changes in average cell size and more subtle changes in their macromolecular composition. We have devised and performed further experiments that allow us to reject certain explanations for these changes. However, we have not provided independent evidence either to confirm or refute other plausible explanations for these changes.

Changes in competitive fitness on substrates other than glucose

The estimates of competitive fitness shown in Figure 1 were all obtained in the same glucose-supplemented

minimal medium in which the experimental populations had evolved. To gain further insight into the phenotypic bases of the derived genotypes enhanced competitiveness, we also examined the fitness of the derived genotypes relative to their ancestor in media in which various other substrates replaced glucose as the sole source of carbon and energy (Travisano et al., 1995; Travisano & Lenski, 1996).

Maltose is a disaccharide of glucose, and the degradation of maltose for energy requires a set of enzymes that are almost identical to those required for the degradation of glucose. But *E. coli* B uses completely different sets of proteins for the uptake of glucose and maltose, as shown schematically in Figure 6. Glucose diffuses through the outer membrane via OmpF porins, and it is then actively transported across the inner membrane via the phosphotransferase system (PTS). By contrast, maltose does not readily diffuse through OmpF porins, but it induces expression of LamB porins that allow maltose (and other maltodextrins) to diffuse into the cell. Maltose also uses an inducible permease, which is not part of the PTS, for its transport across the inner membrane. If the derived bacteria have adapted to the experimental environment primarily by improvements in glucose transport, then one would not expect their higher fitness to be manifest when maltose is substituted for glucose. By contrast, if the derived bacteria have adapted by improving almost any other aspect of their physiology (catabolism, biosynthesis, etc.), then we would expect to find a similar competitive advantage in maltose as in glucose.

Figure 7 summarizes the results of competition experiments between derived and ancestral genotypes in glucose and maltose. All twelve derived genotypes are more fit in glucose than in maltose. The derived genotypes are also much more variable in their competitive fitness in maltose than in glucose. These outcomes support the hypothesis that improvements in glucose transport contribute substantially to their greater fitness.

To further test this hypothesis, we performed competition experiments using a total of twelve different growth substrates. Several of these substrates share with glucose the use of OmpF and the PTS for transport across the outer and inner membranes, respectively, whereas the other substrates differ at one or both steps. To compare the responses to all twelve substrates, we calculated an index of parallel adaptive evolution for each one. The numerator of the index is the proportion of the derived genotypes which are more fit than their ancestor in a given substrate, and the denomina-

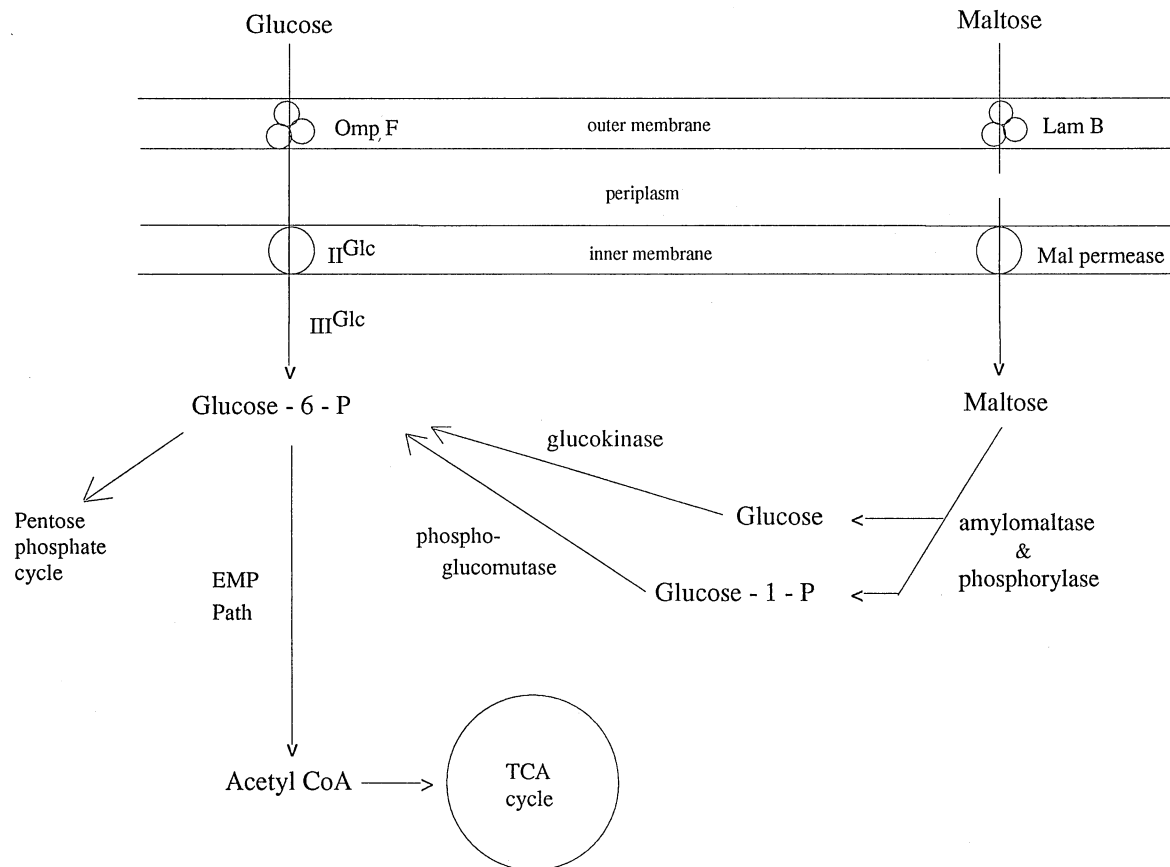


Figure 6. Schematic illustration of the gene products involved in the uptake of glucose and maltose by *E. coli*. Maltose is a glucose disaccharide; glucose and maltose are transported into the cell by completely different mechanisms, but their subsequent catabolism is virtually identical. Reprinted with permission from Trivisano et al. (1994).

tor is the genetic variance in fitness among the derived lines. We expect a higher value of this index for those substrates that are most similar to glucose in terms of the mechanisms that underlie adaptation of the derived genotypes to the experimental environment. As shown in Figure 8, the derived genotypes show the highest degree of parallel adaptive evolution for glucose, followed by the five other substrates that use both OmpF and the PTS for their uptake by the cell; those substrates that differ at one or both steps in transport give the lowest values. These results provide additional support for the hypothesis that improvements in glucose transport are largely responsible for the gains in competitive fitness that occurred during the experimental evolution.

Changes in mutation rates

Spontaneous mutations (including point mutations, transpositions, etc.) provide the genetic variation that is required for evolutionary change in our experimental populations. Therefore, we sought to determine whether the rates of mutation themselves might have undergone evolutionary change (Sniegowski et al., 1997). To that end, we performed a series of fluctuation tests (Luria & Delbrück, 1943) to estimate the rates at which the ancestral and derived genotypes mutate at each of three different loci (at which mutations produce distinct phenotypes that can be readily scored). These experiments indicate a mutation rate of about 10^{-10} per basepair per generation for the ancestral strain, which is similar to values that have been reported for 'wild-type' *E. coli* (Drake, 1991). We obtained similar estimates for nine of the derived genotypes isolated after 10000 generations of evolution. For three of the

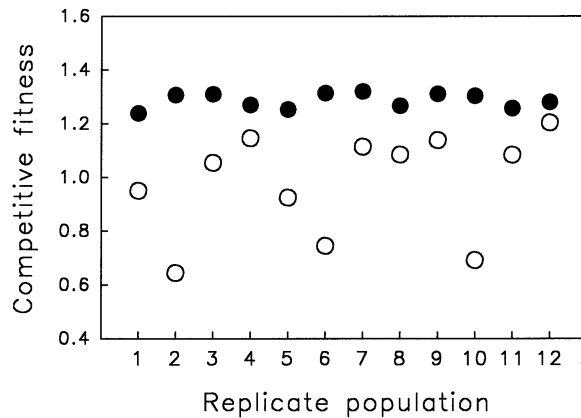


Figure 7. Competitive fitness of derived genotypes from twelve replicate populations of *E. coli*, measured in glucose (filled symbols) and maltose (open symbols), after 2000 generations of experimental evolution in a medium containing only glucose. Fitness is expressed relative to the common ancestor. All twelve lines are more fit in glucose than in maltose. Note also that the derived lines are much more variable in their competitive abilities in maltose than in glucose. Data from Travisano et al. (1995).

derived genotypes, however, mutation rates were estimated to be about two orders of magnitude higher at all of the loci examined. We used samples that had been periodically taken and stored from each population to determine when the mutator phenotype arose and whether it was stably maintained. All three populations with elevated mutation rates had acquired the mutator phenotype by 8000 generations, and each one retained that phenotype for thousands of generations. Evidently, there were dramatic and stable increases in the genomic mutation rate in some, but not all, of the experimental populations.

We have begun to characterize the mutations that are responsible for producing these mutator phenotypes. A number of loci are known that can increase the genomic mutation rate when they are mutated (Cox, 1976; Horiuchi et al., 1989). Genetic complementation tests indicate that increased mutation rates in the three derived genotypes have resulted from defects in the methyl-directed mismatch repair system. But even knowing the genetic basis of the increased mutation rate, one does not know the population genetic process by which the mutator genotypes became established in the evolving populations. On the one hand, a higher mutation rate would be expected to accelerate the appearance of rare beneficial mutations, perhaps giving some long-term evolutionary advantage to genotypes with higher mutation rates. On the other hand, many more mutations are harmful than are beneficial, and so

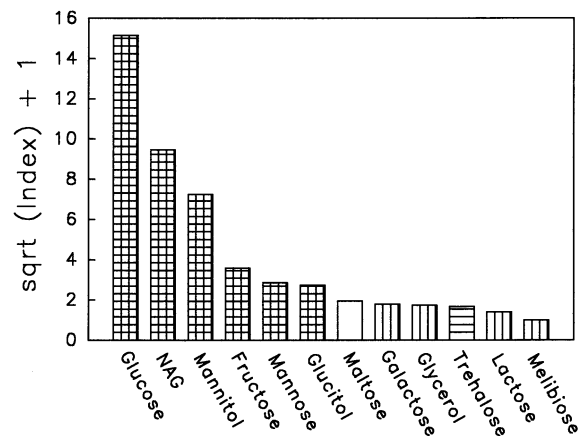


Figure 8. Twelve nutrients are ranked on the basis of an index of parallel adaptive evolution. The numerator of the index is the proportion of the derived genotypes that are more fit than the ancestral strain during competition for that nutrient. The denominator of the index is a measure of the variability in competitive fitness among the derived genotypes. Vertical hatching denotes a nutrient that uses OmpF as its primary mode of transport across the outer membrane, and horizontal hatching denotes a nutrient that uses the phosphotransferase system for transport across the inner membrane. The derived genotypes showed the most parallel adaptive evolution in glucose, followed by the five other nutrients that share with glucose their mechanisms of transport across both membranes. Reprinted with permission from Travisano et al. (1996).

the short-term effect of having a higher mutation rate should be to reduce a genotype's mean fitness. Thus, it would seem to be difficult for mutator genotypes to become established, even if they might provide some long-term advantage (Chao & Cox, 1983).

We can suggest two possible explanations for how mutator genotypes became established in some of the experimental populations, despite their presumably increased load of deleterious mutations. One possibility is that the mutator phenotype is a side-effect of a mutation whose primary effect confers a selective advantage. For example, selection for more rapid growth might have favored the loss of DNA repair functions, because their expression may slow the rate of DNA replication. The other possibility is that, by recurrent mutations, the mutator genotypes reached a sufficiently high frequency (despite their harmful short-term effect) that eventually a secondary beneficial mutation occurred in a mutator background. The mutator gene could then have hitchhiked to high frequency in association with the beneficial secondary mutation (Taddei et al., 1997). Importantly, neither of these hypotheses requires selection for faster evolution *per se*, although both could have that consequence.

It should be possible to distinguish between these two hypotheses by moving the mutator and corresponding 'wild-type' alleles to isogenic backgrounds, and then measuring the short-term effect of the mutator allele on competitive fitness. If the mutator allele prevails during short-term competition experiments, then that would support the hypothesis that the mutator phenotype is a side-effect of a mutation whose primary effect is beneficial in the experimental environment. Alternatively, if the mutator allele is at a competitive disadvantage when placed in isogenic backgrounds in the experimental environment, then that outcome would support the hitchhiking hypothesis.

Conclusions and future directions

Microbial ecologists have long been interested in understanding what attributes make one bacterial genotype or species more competitive than another in a particular environment. Yet it remains unclear how even to address this problem in any general way, despite decades of study devoted to the physiology, biochemistry, and molecular genetics of certain bacterial species, such as *E. coli*. There are many reasons for the difficulty in making substantial progress on this front. Whereas other fields of microbiology have successfully employed a reductionist approach to characterize one gene or pathway at a time, the competitive fitness of an organism may depend on the integration of all its component genes and pathways. Also, natural environments are notoriously variable and unpredictable, making it difficult even to quantify reproducibly the relative fitness of two bacterial competitors, which is essential before one can properly begin to ask why one type prevails over another.

To circumvent these and other problems, we sought to identify what makes one bacterial genotype a better competitor than another by employing a highly simplified experimental system. Twelve populations of *E. coli* were founded from a common ancestor and then propagated for 10000 generations in a defined laboratory environment consisting of serial transfer in a glucose-supplemented minimal medium. During this time, the twelve populations adapted genetically, so that at the end of this period the derived genotypes were demonstrably much more fit in this experimental environment than was the ancestral strain. The derived genotypes grow $\sim 50\%$ faster than does the ancestor when they are placed in direct competition.

We have subsequently characterized the changes that occurred during this experimental evolution in var-

ious traits that might affect their competitive fitness. We showed, for example, that the derived bacteria had achieved a higher maximum growth rate in the experimental environment, and they also experienced a shorter lag phase prior to exponential growth upon transfer into fresh medium. Unexpectedly, we also found that the derived bacteria produce cells that are much larger, on average, than those produced by the ancestral strain. The difference in cell size persists even when the derived bacterial genotypes are forced to grow at the same rate as the ancestral strain, so that it cannot be explained by the well-known phenotypic correlation between growth rate and cell size. The derived bacteria may also have a somewhat higher ratio of total cellular RNA to DNA than does the ancestor; we rejected one possible explanation for this change, which was that the derived bacteria had acquired an additional copy of an *rrn* operon.

We also asked whether the advantage enjoyed by the derived bacteria persisted when the competition experiments were performed in eleven other environments in which glucose was replaced by different substrates. In general, the derived genotypes had higher and more uniform competitive abilities for substrates that share with glucose the mechanisms of their transport into the cell; whereas the derived genotypes were less fit and more heterogeneous when competing for substrates that use other mechanisms of transport. These findings suggest strongly that changes in glucose transport involving the porins, the phosphotransferase system, or both, contributed to the improved competitive fitness of the derived bacterial genotypes. [See also Dykhuizen & Dean (1990) for a review of some other experiments which provide compelling evidence that transport functions often limit bacterial growth.] Another unexpected change in three of the twelve derived populations is that the mutation rate has increased by some two orders of magnitude. The increased mutation rate is a genome-wide effect, and in each of the three cases it is caused by defects in the methyl-directed mismatch repair system. We do not yet know why these mutator genes increased in frequency. One possible explanation is that the bacteria were able to grow faster by disabling this repair process; another possibility is that the mutator gene 'hitchhiked' along with some beneficial mutation at another locus. In any case, these changes in mutation rate may affect (for better or for worse) not only the competitiveness of the derived bacteria but also the further evolution of their competitiveness.

Evidently, we have had some success in identifying a number of phenotypic traits that have changed as the bacteria have adapted to, and become better competitors in, the environment in which they were propagated for 10000 generations. Some of the changes seem to contribute quite directly to the higher competitive fitness of the derived bacteria, for example, enhanced glucose transport and a shorter lag phase upon transfer into fresh medium. But in other cases, it is unclear how the phenotypic changes contribute to the competitive success of the derived bacteria. Do large cells have an intrinsic competitive advantage over smaller cells and, if so, why? Or is a larger cell size simply a correlated consequence of some physiological change that, once identified, will be readily understood as contributing to improved fitness? For example, improvements in glucose transport might cause an increase in average cell size merely as a consequence of changing the relative rates of resource uptake and genome replication. Similarly, have mutation rates increased in some of the evolving populations because disabling a system of DNA repair allows faster growth, or for some other reason?

Clearly, our findings have raised at least as many questions as they have answered. We are optimistic that we can, in fact, address these questions. We now have two sets of candidate loci that can be compared between the derived and ancestral genotypes, those involved in glucose transport and those encoding the methyl-directed mismatch repair system. Once we have identified precisely the genetic changes that occurred during the experimental evolution, we can then perform genetic manipulations to construct isogenic strains that will enable us answer the questions posed above. For example, we might find that the same mutations that enhance glucose transport also increase cell size as a side-effect, thus obviating the need for some separate adaptive explanation for the evolution of larger cells. Similarly, we might find that the mutations causing defects in methyl-directed mismatch repair allow more rapid growth, thereby providing them with a direct selective advantage in the experimental environment.

Thus, the 'top-down' approach that we have used to characterize changes in phenotype that took place during the experimental evolution has generated new hypotheses that can be tested using a 'bottom-up' approach in which specific genes are manipulated. Ultimately, a combination of these two approaches may reveal whether the competitive fitness of bacteria can be understood by examining one trait (or one

gene) at a time, or whether it is essential to study bacteria in a more integrated fashion in order to understand their ecological capabilities.

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