EXPERIMENTAL EVOLUTION

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Evolution is considered as the most fundamental process in biology, connecting all life by what is called the common ancestor. Since Darwin proposed the idea of evolutionary adaptation by natural selection, many theoretical and comparative studies have been performed to elucidate the underlying processes. But in the last decades experimental evolution allowed us to study the evolutionary dynamics as they happen, by directly monitoring microorganisms that propagate in an experimental environment. Here, we aim to comprehend the insights that these studies gave in the process of evolutionary adaptation by natural selection. The longest-running microbial evolution experiment was started in 1988, at which twelve populations were founded by the same strain of *Escherichia coli*. In this, and other evolution experiments, adaptive radiation was observed in both spatially and temporally heterogeneous environments. The most fundamental process in maintaining diversity was shown to be frequency-dependent selection. In addition, the long-term experiment showed a remarkable parallelism in phenotypic changes that were observed in the twelve independently evolving populations, which is a hallmark for adaptive evolution. These parallelisms were affecting two different levels of gene regulation, namely global regulatory networks and local regulons. Especially two highly interconnected networks – genes that regulate DNA topology and the stringent response – have been demonstrated to be involved in the phenotypic and genetic adaptation. The dynamics of this evolutionary adaptation could quite readily, although not completely, be explained by the observed beneficial mutations that were substituted, in which the roles of natural selection and clonal interference seem to be the most dominant. These dynamics, together with the discovery of many epistatic interactions, demonstrated that the adaptive landscape was quite rugged. In general, experimental evolution has provided one of the most straightforward evidences for adaptive evolution by natural selection.

INTRODUCTION

When Darwin (1858, 1859) postulated his theory of evolutionary adaptation by natural selection, not much was known about the extended diversity of microbial life. Also later in the history of evolutionary research, like in the Neo-Darwinism (Mayr, 1991; Weismann, 1892) and Modern Synthesis (Mayr, 1991), theories were almost exclusively deduced on observations of eukaryotic and *bisexual* [see Glossary] life (Kutschera & Niklas, 2004). Although the role of microorganisms in the origin of the evolutionary theory was minor, their role in biological evolution itself was not. Microorganisms were already present more than ~4 billion years ago (Schopf, 1993) and Carl Woese and colleagues (1990) showed that two of the three domains in the tree of life solely consist of microbial life. Evolutionary biology has relied primarily on comparative studies of living organisms, supplemented by the fossil record and, recently, by phylogenetic data. But all these approaches study evolutionary dynamics indirectly and much uncertainty about genetic variation and dynamics of the past therefore exists. It is only in the last decades that a new approach allows phenotypic and genotypic evolution to be observed in action, namely by experimental evolution. In experimental evolution, microorganisms (but also fruit flies, mice and other organisms) are used in simple experimental environments to study evolution by directly observing changes in pheno- and genotypic characteristics. Pioneering work by Atwood and colleagues (1951) was done in the early 1950s but, except in the fruitfly school, this approach did not take hold. It was not until the early 1990s that the importance and impact of experimental evolution studies with the aid of microorganisms was fully recognized (Dykhuizen, 1990). Over the last decades this approach has been widely used for explaining evolutionary adaptation by natural selection (Elena & Lenski, 2003; Lenski & Wiser, 2009). In this study, we aim to comprehend the insights gained by experimental evolution in the dynamics of evolutionary adaptation and divergence.

The use of microorganisms in experimental evolution studies has multiple benefits. Firstly, they have short generation times, which allow experiments to run for thousands of generations. However long-term experiments that run for thousands of generations

are, in the evolutionary perspective, still very short. A second advantage is the large population size, which provides a tremendous supply of *de novo* mutations that generate the genetic variation needed for evolutionary adaptation. Third, ancestral and derived samples can be frozen and revived later, so that their phenotypes can be simultaneously measured, together with their relative fitness by head-to-head competitions. In this competition experiment the ancestral and derived lines can be discriminated by the use of a neutral marker. The benefit of using an asexual organism is that these markers are linked with the entire genomic background. And lastly, there are abundant molecular and genomic data for many microbial species, as well as molecular techniques for genetic analysis and manipulation.

Evolutionary adaptation by natural selection, as described in Darwin's book "The Origin of Species" (1859), logically follows from three simple premises. First, individuals within a population may vary from each other in multiple phenotypic characteristics. Second, these phenotypic characteristics contribute non-randomly to an individual's fitness in terms of survival and reproduction (survival of the fittest). And lastly, the phenotypic characteristics are heritable, at least in part. Hence, individuals in later generations will tend to be better adapted to their environment than individuals in earlier generations. Evolution is thereby the change in the genetic composition of a population over generations. In general, the four fundamental processes that contribute to evolutionary change are mutation, *mixis*, natural selection, and *genetic drift*. Mutation and mixis produce the heritable variation that can be selected by the environment (natural selection) or randomly sampled over generations (genetic drift). In this review, only the variation that is produced by *de novo* mutations will be considered (excluding mixis by horizontal gene transfer). Therefore, the experimental results considered in this review are the product of mutations, natural selection and genetic drift. In terms of these general processes, there are a few remarks that have to made when focussing on experimental evolution. Firstly, experimental evolution considers natural selection and not artificial selection. In other words, in experimental evolution the individuals that contribute to the next generations are not selected by a researcher on the basis of their phenotypic characteristics, but are blindly picked, whereby the genotypes that are most numerous have the highest chance in contributing to the next generation (in the case of a serial regime). So, the three premises necessary for adaptive evolution by natural selection still hold in experimental evolution. Secondly, the advantage of experimental evolution studies is that the evolutionary process is studied outside its natural and complex ecological environment, like Darwin (1859) stated, "Evolutionary change occurs within an ecological context, but the workings of that context can be infinitesimally complex". So, with experimental evolution the ecological complexity is reduced by using monocultures in simplified environments. It is this deliberate reduction of complexity that is the strength of experimental evolution. Like Mendel's (1866) study, on the heredity of discrete traits in a single simplified system (garden peas), served as a framework for quantitative genetics in explaining complex continuous traits, experimental evolution can form a framework for studying evolutionary adaptation in its complex ecological environment.

This review is subdivided in three main parts. Firstly, *polymorphism and diversification* where the origin and maintenance of phenotypic diversity will be discussed. This is a logical first step in explaining the underlying processes of the origin and existence of biodiversity. Then, we review *the multiple levels of evolutionary adaptation*, where parallel changes on the genomic level, in RNA expression and in protein profiles will be discussed; such parallelism is seen as a hallmark of evolutionary adaptation. And, in the end, *evolutionary dynamics* will be discussed, such as the differences between genomic and phenotypic evolution, clonal interference, the extent of between-population variation, etc. In summary, we thus aim to comprehend insights gained by experimental evolution studies on evolutionary adaptation by natural selection. Most of the work considered here comes from the longest running evolution experiment in *Escherichia coli* performed by Lenski and colleagues (1991; in the rest of the paper referred to as, the long-term experiment). This study showed some outstanding results and thereby forms one of the most robust experimental evidences for the existence of Darwinian evolution. Before we continue with the above described paragraphs, we will first give a short introduction in the experimental setup, fitness measurements and mutation rates of Lenski's long-term experiment as a basis for the rest of this review.

Setup of long- term experimental evolution in Escherichia coli

Experimental design and conditions

In total, 12 replicate populations were founded from the same ancestor and consequently populations were isogenic except for a single marker that will be described below. So, at the start of this experiment there was no genetic variation (other than that of the inoculum), neither within and between the different replicate populations, hence most variation that eventually arose was from *de novo* mutations. Likewise evolution was entirely dependent on these new mutations (Lenski *et al.,* 1991). The 12 populations were divided in two groups of six populations in accordance with their neutral marker. One group was unable to catabolize the sugar arabinose (Ara^-), and the other group, thanks to a point mutation in the *ara* operon, was able to grow on arabinose (Ara^+). The Ara^- and Ara^+ clones formed red and white colonies, respectively, when they were

plated on tetrazolium arabinose (TA) plates (Levin *et al.*, 1977). This made it possible to distinguish two genotypes, when put together in a head-to-head competition experiment, to measure their relative fitness (see below). In this same way, evolutionarily derived genotypes were allowed to compete with the ancestral strain of the opposite marker state. Thereby, relative fitness could be measured directly, of course with the assumption that evolution was transitive. It was also used to control for cross-contamination, namely all lines were propagated by alternating between Ara^- and Ara^+ lines. Therefore, if there was cross-contamination this could immediately be noticed by looking at the marker, this to guarantee that the observed parallel evolutionary changes were evolutionarily independent. The engineered marker was selectively neutral for the environmental conditions in this experiment (Elena & Lenksi, 2003; Lenksi *et al.*, 1991).

The twelve populations were serially propagated at 37 ^oC in Davis minimal medium supplemented with 25 µg of glucose per mL. Each day, 0.1 mL grown culture of the previous day was transferred into 9.9 mL of fresh medium. This 100-fold dilution allowed for ~6.64 [$^2\log(100)$] generations of binary fission per day, fluctuating between ~5 x 10^6 and ~5 x 10^8 cells per 10 mL culture (Lenski, 2004). This means ~2.400 generations a year (which is an incredible number compared to 'non-microbial' organisms). And when you consider that this experiment was started on 15 February 1988 and is still running, the cultures have been evolving for more than 50.000 generations. The environmental conditions, with as the sole carbon source glucose and fluctuations between 'feast' and 'famine' in accordance to the glucose concentration, were encountered by the ancestral bacteria for the first time. This allowed enough 'space' for evolutionary adaptation to occur.

Of all evolving populations, samples were stored every 100 generations and later every 500 generations. These samples were stored at -80 ^oC, with glycerol added as a cryoprotectant. The samples have always been readily able to revive bacteria and there were no signs of mutational events during their storage (Lenski, 2004). Before making any measurements, the samples of interest were removed from the freezer simultaneously followed by a period of acclimatization. In this way, all measurements were performed under the same conditions, to be sure that only the heritable differences could be observed.

Fitness measurements

As explained in the previous paragraph, fitness measurements were performed by using the neutral marker (Ara^- competitor *vs.* Ara^+ competitor). This way, it was possible to compare the relative growth rate of each competitor by counting the colonies before and after the competition experiment (which occurs in a liquid environment). The relative fitness was defined as the ratio of the two competitors' [*i* and *j*] net growth rates, which is equal to the ratio of their Malthusian parameters, m_i and m_j:

$$W_{ij} = \frac{m_i}{m_j} = \frac{\ln\left(\frac{N_{i,1}}{N_{i,0}}\right)}{\ln\left(\frac{N_{j,1}}{N_{j,0}}\right)} = \frac{\ln\left(\frac{N_{i,1}}{N_{i,0}}\right)\Big/\ln(2)}{\ln\left(\frac{N_{j,1}}{N_{j,0}}\right)\Big/\ln(2)} = \frac{^2\log\left(\frac{N_{i,1}}{N_{i,0}}\right)}{^2\log\left(\frac{N_{j,1}}{N_{j,0}}\right)} = \frac{D_i}{D_j}$$

(*N* = population size *or* number of colonies; subscript 1 and 0 represent, respectively, conditions before and after the competition experiment) , the relative fitness (W_{ij}) is similar to the relative number of doublings (D_i/D_j) and the relative Malthusian parameters (m_i/m_j). These calculations assume that no cells were lost and therefore a death rate of zero, which was also proven to be the case (Vasi *et al*., 1994).

Mutation rates

Because most genetic variation in these experiments is produced by *de novo* mutations, it is interesting to see how likely and often mutations in these populations occurred. The number of mutational events is simply the product of genome size, population size, mutation rate and the number of generations (doublings) (Lenski, 2004). Like described above, the population size fluctuated between ~5 x 10^6 and ~5 x 10^8 cells per 10 mL culture. The *effective population size*, as the population size that produces the mutations, is approximately equal to the bottleneck population times the number of generations between the minimal and maximal population sizes: N_e = 5 x 10^6 x $^2\log(100) \approx 3 \times 10^7$. The mutation rate in *E. coli* has been estimated to be about 1 x 10^{-10} per base pair (Lenski *et al.*, 2003). The genome length of *E. coli* is about 5 x 10^6 base pairs (Blattner *et al*., 1997). In the latest articles the number of generations that are considered is 20,000, therefore the expected number of mutations per population is 3 x 10^8. Considering the genome size, this is equal to more than 50 mutations per base pair, this even after adjusting for the transfer bottleneck and using a relatively low expected mutation rate (in comparison with Drake *et al*., 1998). This mutation rate is only accounting for the point mutations, so chromosomal rearrangements like insertions, deletions and inversions are not considered. Although the mutational input provides a tremendous amount of variation, it does not mean that every possible sequence has ever existed. Far from this, because each mutation occurs against relatively few genetic backgrounds (each base pair position has around 50 mutations, this means that the maximal possible genetic backgrounds for a mutation at this specific position is also around 50).

The mutational redundancy, and possibly the redundancy of beneficial mutations, does not immediately guarantee a high substitution rate. Firstly, most beneficial mutations are lost by genetic drift (Haldane, 1927). And secondly, beneficial mutations

occurring in different genomic backgrounds will outcompete each other by *clonal interference* (Gerrish & Lenski, 1998; both genetic drift and clonal interference will be discussed in more detail in the paragraph 'Evolutionary dynamics'). Lenski (2004) estimated that in total only between 10 and 20 beneficial mutations have occurred in each population over these 20,000 generations. This seems to be extremely low in comparison to the number of point mutations which was several hundreds of millions in each population (as calculated above). Combining the beneficial and neutral substitutions, in total fewer than 100 substitutions would be expected (Lenski, 2004), this does not account for the number of other chromosomal rearrangements and hypermutable regions. In conclusion, this shows that the seemingly extensive variation that is produced *de novo* seems to result in a rather low number of substitutions. The studies which we will discuss below, successfully attempt to discover these substitutions and map them to their phenotypic characteristics. The low number of expected substitutions also confirms that 20,000 generations in the light of evolution is still very short, however evolutionary and ecological timescales are often considered most closely related and overlapping in microorganisms.

POLYMORPHISM AND DIVERSIFICATION

The key aspect, when examining diversity incurred by adaptive radiation, is species interactions. But, in hindsight, it is often hard to assess which interactions are responsible for the biodiversity we observe today. For example, competition can either promote or prevent adaptive radiation, depending on the relative benefits of specialization *vs*. general adaptation. Darwin (1859) partly circumvented this problem by considering adaptive radiation on isolated islands. Here, ecological interactions could be investigated directly and tell something about the possible processes underlying the observed biodiversity (when assuming that the observed biodiversity evolved from a single founding species). Of course, with experimental evolution, it is possible to observe these interactions directly during the evolutionary process of diversification, which makes a comparative approach, for evaluating the ecological interactions in hindsight, unnecessary (Rainey *et al.*, 2000; Travisano & Rainey, 2000). The ecological complexity needed for adaptive radiation is well explained by the *niche exclusion principle* (or *competitive exclusion principle*). Early work of Gause (1934) on *Paramecium* showed that "the number of species cannot exceed the number of distinct resources", which is better known as the *competitive exclusion principle* (Gause, 1934; Hardin, 1960). Although a species' niche is not only defined by a particular resource, as Hutchinson (1958) denoted, a niche is an "n-dimensional hypervolume" of ecological variables. The *niche exclusion principle* therefore states that ecological opportunities are needed before adaptive radiation can occur, although the existence of such opportunities is no guarantee for diversification. In the next sections we examine two forms of ecological heterogeneity, namely *spatial* and *temporal heterogeneity*, and show that even in the simple ecological environments of these experiments, diversification is possible. Then we will discuss the processes underlying these diversification events.

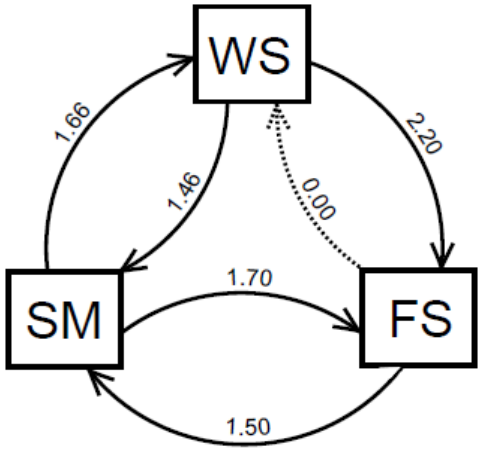


Figure 1. Competitive relationships among niche-adapted classes. Arrows point from the initial rare morph to the common morph for each of the competitions. Fitness measures were determined by the ratio of Malthusian parameters (see introduction) of the initially rare genotype to the common genotype, so that a fitness of 1.00 indicates genotypes of equal competitive ability. From Rainey & Travisano (1998).

Spatial heterogeneity

In multiple studies it is shown that spatially structured environments facilitate diversification. One of the most striking examples of adaptive radiation by spatial heterogeneity is given by the study of Rainey and Travisano (1998). They propagated a single ancestral genotype of *Pseudomonas fluorescens* with and without shaking the culture, so varying the opportunity for environmental gradients to be formed. Propagation without shaking, and therefore with spatial heterogeneity, resulted in diversification. From the ancestral smooth colony morph (SM), wrinkly spreader (WS) and fuzzy spreader morphs (FS) evolved. In the shaking conditions only the ancestral morph could be found. When the diversified culture, in the non-shaking condition, was switched to the shaking condition, diversity was quickly lost. So this clearly showed that spatial heterogeneity was needed for both the origin and the maintenance of diversity (the differentiation was genetically based and the experiment continued for only 10 days).

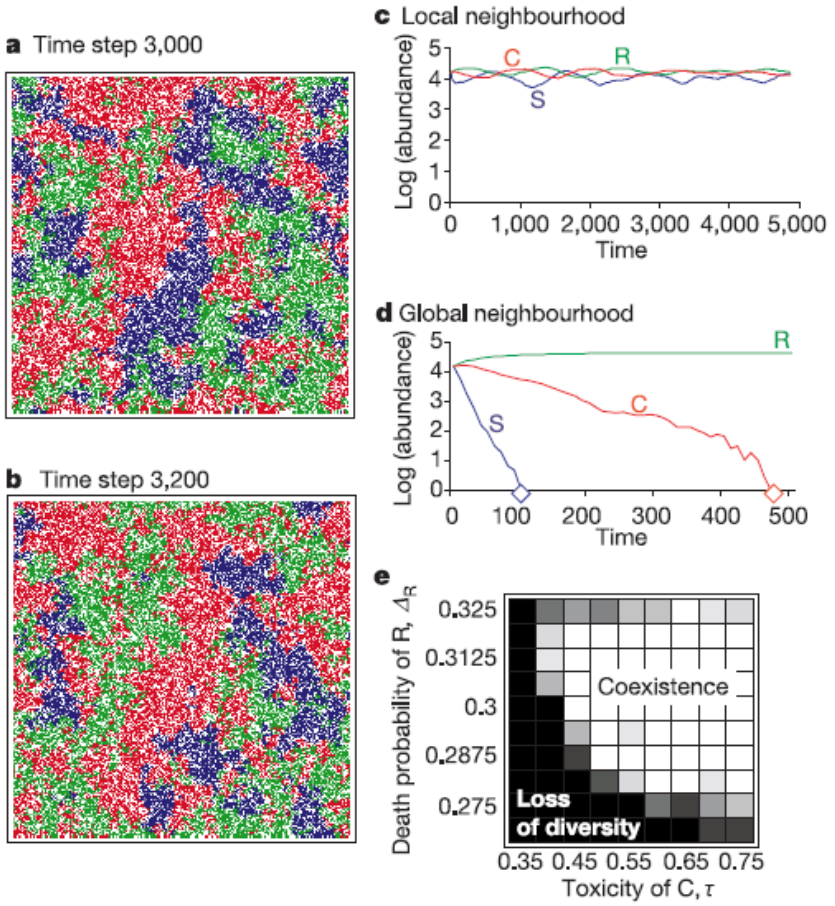


Figure 2. Predictions of the latticed-based simulation, see Kerr *et al*. (2002). Colour-code is as follows: C = colicinogenic cells, *red*; R = resistant cells, *green*; S = sensitive cells, *blue*. **a**, **b**, Snapshots of the lattice in a simulation with a local neighbourhood at times 3,000 (**a**) and 3,200 (**b**), for details about the time unit see Kerr *et al*. (2002). **c**, The complete community dynamics for the same simulation run (abundance is log-transformed). **d**, Community dynamics for a simulation with a global neighbourhood, in other words, without spatial locality (abundance is log-transformed). When a strain goes to zero, it is represented as a diamond on the abscissa of the relevant graph at the relative time. See, for the used parameter specifications Box 1 in Kerr *et al*. (2002). **e**, Sensitivity of the qualitative dynamics to changes in a subset of parameter values. The grey scale indicates the number of 'local' simulated runs in which coexistence occurred for at least 10,000 time units, with a lighter area indicating a higher probability of coexistence. Toxicity of C, is the increased death rate of S thanks to a neighbouring C cell. Death probability of R, chance that R is killed, this value is not influenced directly by its neighbours. In the simulations it is assumed that the death rate of R is bigger than that of S. (Kerr *et al*., 2002)

If selection caused the observed diversification you would expect competitive trade-offs between the different niche-adapted morphs. This was tested by competing the different morphs against each other in a pairwise fashion (SM versus WS, SM versus FS, FS versus WS). To check for frequency-dependent selection the ratio of competing morphs at the start of each competition event was 1:100. Figure 1 shows that five of the six interactions were frequency dependent, in which the rare morph had a selective advantage over the more abundant one. So competitive trade-offs were indeed responsible for the maintenance of polymorphism, which implies that competition plays an important role in adaptive radiation. Because FS was unable to invade WS, its maintenance was probably dependent on interactions with all three morphs. The persistence of three different morphs in this spatially structured environment shows that there were at least three different avenues for adaptation (adaptive pathways) for the ancestral genotype. There were two additional striking aspects in the adaptive radiation observed in this system. First, the complex ecological interactions evolved in a very short period of time, after three days. Therefore, after only tens of generations, the distinct morphs were already visible. Second, the different morphs were differentially located in accordance with their growth pattern: WS morphs mainly occurred at the air-broth interface, SM morphs were most abundant in the broth, and FS morphs at the bottom. This spatial pattern implies that these morphs evolve by niche specialization to the local microenvironments, although the exact physiological adaptation is unknown.

Kerr and colleagues (2002) performed another interesting study on diversity. They studied the role of a spatially structured environment in maintaining a non-hierarchical system of three different *E. coli* strains. One strain produced the toxin colicin (C), another strain was resistant to this toxin without producing it (R) and the last strain was sensitive to colicin (S). In this way, a rock-paper-scissor game was formed, in which C displaced S (C kills S), R displaced C (growth advantage, cost for producing the toxin) and S displaced R (growth advantage, cost for being resistant). So, in contrast to Rainey and Travisano (1998), not the origin but the maintenance of diversity was tested in a system of which the exact ecological interactions were known. A computer simulation showed that the different strains could only coexist in a spatially structured environment (figure 2). In a mixed system the continual redistribution of C rapidly drove S to extinction, after which R could displace C (figure 2). To test this hypothesis, an experiment was performed in which the three different strains were propagated on a (i) static plate, (ii) shaking flask or (iii) mixed plate (an environment intermediate between these two extremes). And indeed, like shown with the computer simulations, the three strains could only coexist when enough spatial heterogeneity was present (figure 3). It is important to realize that the spatial heterogeneity, in this case, is not caused by environmental gradients but by local

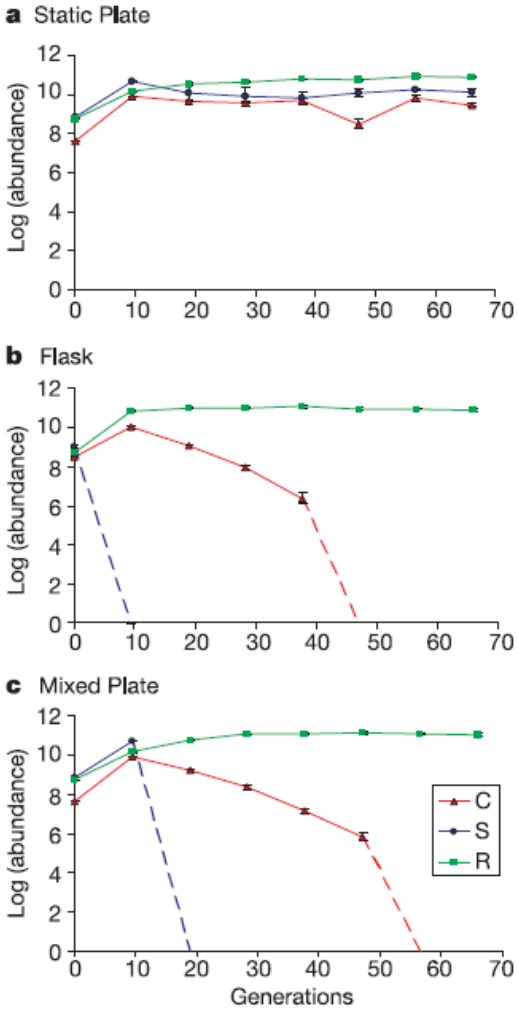


Figure 3. Community dynamics in the experimental treatments (Kerr *et al*., 2002). **a**, Static plate; **b**, Flask; and **c**, Mixed Plate. Dashed lines indicate that the abundance of the relevant strains has decreased below its detection point. Data points are the mean of three replicates, and bars indicate standard error of the mean. Data points are separated by approximately 10 bacterial generations. From Kerr *et al*. (2002).

biotic interactions. The importance of locality in the maintenance of variation, even without abiotic heterogeneity, shows that in studying evolutionary processes not only the different ecological interactions are important but also how they are distributed in space.

In conclusion, spatial heterogeneity seems to play an important role in adaptive radiation. Spatial heterogeneity results in niche specialization, in which specific genotypes have local competitive advantages as a consequence of their physiological adaptations. The locality of these competitive advantages results in an overall frequency-dependent selection and therefore the maintenance of a stable polymorphism (in a later paragraph the selection pressures that could be responsible for the maintenance of diversity will be discussed). However, in both studies the dynamics are only tested for a rather limited amount of time, so the stability of the observed polymorphisms is still questionable.

Temporal heterogeneity

However, we quoted Gause (1934) in the introduction of this paragraph, with the phrase that the number of species can never exceed the number of resources. It is imaginable that variation in one resource leading to periods of 'feast' and 'famine' can result in the emergence of specialists and therefore diversification. This is most easily seen using a theoretical example. Imagine that the growth of each population is given by (Monod (1949) resourced-based model of bacterial growth):

$$\frac{dN}{dt} = N \cdot g(S) = N \cdot \frac{V_{\max} \cdot S}{S + K_s}$$

where N is cell density, g(S) is growth-rate function which is dependent on substrate concentration, V_{max} is maximal growth rate, S is substrate concentration and K_s is resource concentration at which growth is half the maximum rate. So, if $S \rightarrow \infty$ than $g(S) = V_{max}$ and if $S = K_s$ than $g(K_s) = \frac{1}{2}V_{max}$. Stable coexistence is possible if one genotype is specialized on the 'feast' period and the other on the 'famine' period. During 'feast' the resource concentration is high (↑S) and the saturation function approaches the maximal growth rate ($g(S) \approx V_{max}$), therefore the 'feast' specialist must have a higher maximum growth rate (V_{max}) than does the 'famine' specialist. In contrast during 'famine' the resource concentration is very low (↓S) and the function becomes linearly dependent on substrate concentration ($g(S) \approx (V_{max}/K_s) \cdot S$), therefore the 'famine' specialist must have a higher substrate affinity (V_{max}/K_s). This example thereby shows that it is theoretically possible to have two coexisting genotypes on one resource as a consequence of temporal dynamics (Travisano & Rainey, 2000).

In the long-term experiment, 'seasonal' variation in glucose concentration occurs by serially propagating the bacteria cultures. Within a new culture derived from this long-term experiment [Ara^{-3}], Souza and colleagues (1997) discovered a stable coexistence of two strains, which was maintained by frequency-dependent selection (although this was not the objective of this study). The two strains differed from each other in several respects, including their ability to utilize the sugar lactose, forming the Lac^- and Lac^+ variant (Turner *et al.*, 1996). This difference in lactose metabolism was used to identify the different strains, but the experiment itself occurred in glucose based medium. To see if the *demographic trade-off*, discussed above, was responsible for the frequency-dependent selection that was observed, the different parameter values (V_{max} and K_s) were measured. And, indeed, a *demographic trade-off* was detected, one strain (Lac^-) having a higher growth rate, and the other (Lac^+) a higher affinity for the substrate.

However, when considering the culture conditions used in the selection experiment, the *demographic trade-off* only marginally contributed to the stable coexistence (Turner *et al.*, 1996). As an alternative, the contribution of *cross-feeding* to the maintenance of the stable coexistence was examined. The metabolites excreted by the faster growing Lac^- strain were efficiently used by the Lac^+ strain, which supported its growth after glucose was depleted. Therefore, the advantage of the Lac^+ strain during the 'famine' period counterbalanced the growth advantage of the Lac^- strain during the 'feast' period. Thus, in this case, adaptive radiation was supported by two different ecological interactions, competition and facilitation, both involving the seasonal nature of the environment. Whereas the relative growth advantages in a spatially structured environment were different over space, in temporal heterogeneity they were different over time.

After 10,000 generations, six of the twelve long-term experiment populations were exhaustively examined for niche specialization (Elena & Lenski, 1997). In one of these populations [Ara^{-2}] disproportionately strong frequency-dependent selection was observed, with a ~7% fitness advantage for a subpopulation when being rare in comparison to ~1% advantage for those in other populations. The underlying mechanism responsible for this strong frequency-dependent selection was tested at 18,000 generations by Rozen and Lenski (2000). They noted the presence of two morphs, one morph (S) producing smaller individual cells, as well as colonies, than the other (L). The mechanisms contributing to the frequency-dependent selection were very similar to those in the Lac^+ and Lac^- strains (Turner *et al.,* 1996). Namely, L grew faster during the exponential growth phase, while metabolites secreted by S and L promoted growth in S (*cross-feeding*). In addition, the presence of S enhanced the death rate of L during the stationary phase. However, the most interesting aspect of this study was the complex dynamics of this coexistence observed when monitoring thousands of generations (figure 4a). The coexistence began at about 6,000 generations and the ratio of the S and L types fluctuated ~60-fold over the thousands of generations that followed (figure 4a and 4b), this in contrast to clones that were isolated at one point in time and immediately converged on a stable equilibrium (Rozen & Lenski, 2000).

To see which mechanism was responsible for the extreme fluctuations, the phylogenetic history and ongoing adaptations of both the S and L type were examined (Rozen *et al.*, 2005). Phylogenetic analysis revealed that both L and S were monophyletic groups (or clades) (figure 5). Two different approaches were used to examine the possible ongoing adaptation within each clade. First, they measured the genetic variation within each clade. If there was continues adaptation, one would expect that selective sweeps cause variation to be purged periodically. And indeed four significant drops in the amount of variation were measured, while only using 1,000 and later 2,000-generation intervals (figure 4c). The second way to

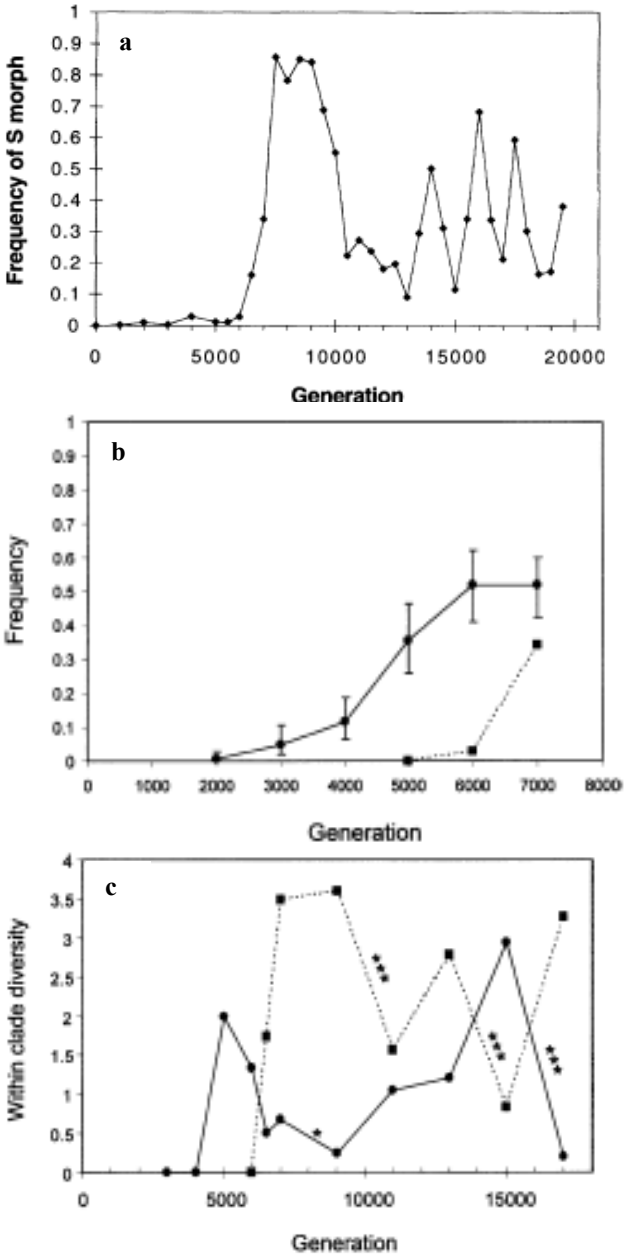


Figure 4. Long-term dynamics of stable polymorphism between S and L clades, see Rozen & Lenski (2000) and Rozen *et al*. (2005). **a**, Each point shows frequency of the S clade, obtained by scoring several hundred bacteria as S or L on the basis of their resulting colony morphology. Despite the short-stability of the polymorphism, it is unstable over much longer intervals. **b**, Trajectory of the appearance and early dynamics of the S and L clades. Frequencies based on sampling among hundreds of clones between generation 2,000 and 7,000. The data for L is shown as circles and solid lines; the data for S are shown as squares and dashed lines. Error bars for L are 95% confidence intervals based on the binomial distribution. **c**, Trajectories of genetic diversity within the S and L clades. The data for L is shown as circles and solid lines; the data for S are shown as squares and dashed lines. Significant declines in genetic variation between consecutive samples are indicated by asterisks: ***$p < 0.001$; *$p < 0.05$. Adapted from Rozen & Lenski (2000) and Rozen *et al*. (2005).

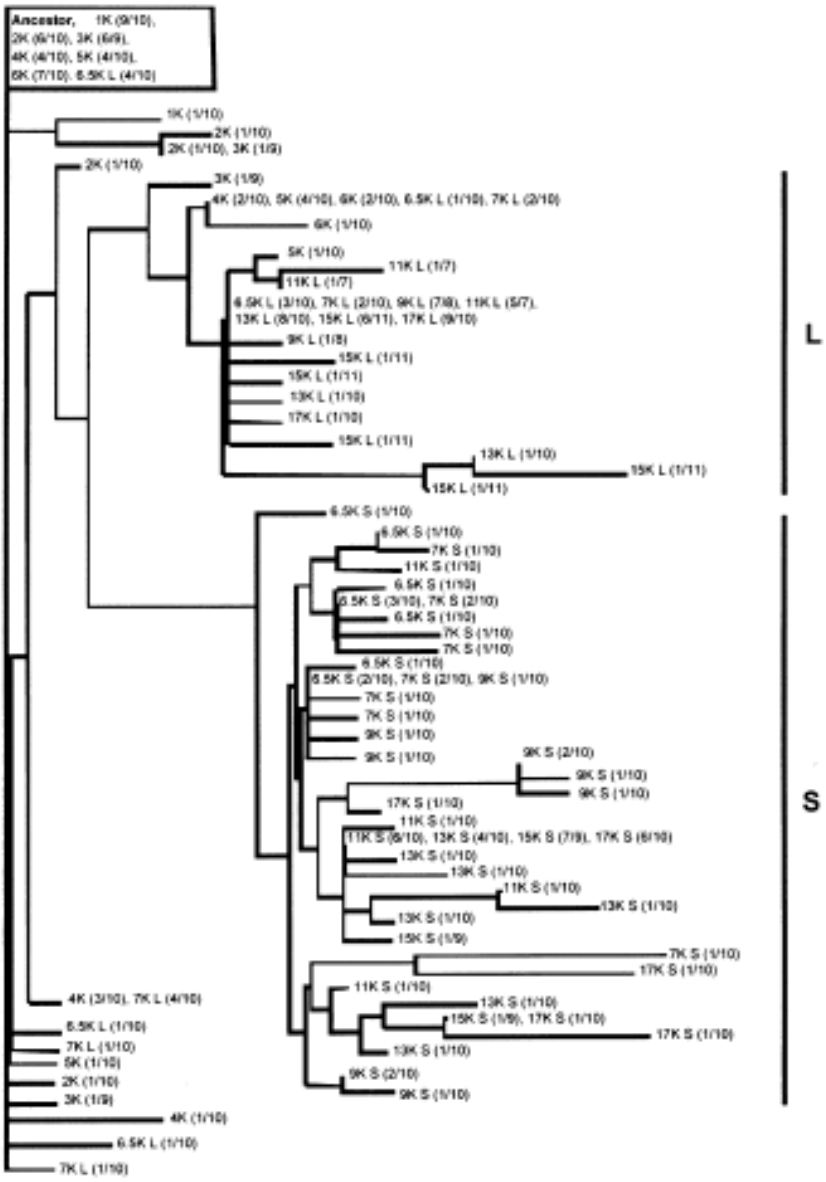


Figure 5. Neighbour-joining tree constructed from RFLP-IS data for hundreds of clones sampled from an evolving, polymorphic *E .coli* population [Ara^{-2}]. The tree was rooted using the actual ancestor genotype, shown at the upper left in a box (along with later clones that had the same RFLP-IS fingerprint). Each genotype shown on the tree is described by generation, morph, and fraction of clones from that generation and morph with the same genotype. The tree was constructed without regard to phenotypic data. Nonetheless, all clones with the S morphology fall in a monophyletic clade, denoted S, indicated by the lower bar at right. All other clones had the L morphology, including the set of L *sensu stricto* that forms the L clade, denoted by the upper bar at the right. The other L clones are assigned to a basal paraphyletic group. Adapted from Rozen *et al*. (2005).

test for continuous adaptation was by allowing clones from an earlier generation to compete with those that derived later. And also with this approach continuous adaptation was proven, whereby the relative fitness of '17,000-generation' clones compared to '13,000-generation' clones is approximately 2% higher within both clades. Rozen and colleagues (2005) provided two alternative hypotheses for these fitness gains. First, it would be possible that both S and L experienced continued improvements in their ability to exploit abiotic environmental opportunities. Alternatively, S and L may be coevolving as a result of them competing for the same resource, glucose, and other secondary metabolites that were excreted. So, the complex dynamics is caused by continuous adaptation of both groups and is contrasting to the polymorphic equilibrium that is stable over short timescales (Rozen & Lenski, 2000). This study is one of the first that show the complexity of eco-evolutionary dynamics, in which the ecological interaction changes as a consequence of ongoing 'within-clade' adaptation. An interesting next step would be to examine the different substitutions causing the selective sweeps and how these mutations change the ecological interaction (maximum growth rates or *cross-feeding*). Also, it would be interesting to know which physiological changes result in the divergence of the S type from the L type around 6,000 generations.

Polymorphism and speciation

The pattern of adaptation and divergence of the L and S type in the previous section, is very similar to the mathematical analyses of adaptive speciation (Dieckmann & Doebeli, 1999; Doebeli & Dieckmann,

2000), in which the ancestral lineage first adapts to the available resource and then divergence into multiple specialists occurs by the partitioning of resources. Of course, the underlying mechanisms of these models (where multiple different ecological interactions are tested) are not exactly the same as those under the experimental conditions (where both *competition* and *facilitation* by *cross-feeding* is involved), but the similarity in the observed dynamics raises the question if this diversification event is an early stage of speciation. The problem with defining a speciation event is the lack of consensus in the species definition for asexual microorganisms. Thanks to the *Biological Species Concept* (Mayr, 1944), which defined a species as a group of interbreeding populations that were reproductively isolated from other such groups, a species in sexually reproducing organisms was viewed as a fundamental unit of ecology and evolution. However, the *Biological Species Concept* does not apply to asexual organisms, like bacteria. Many definitions for asexual species are merely operational and are based on both genetic distance and phenotypic clustering. However Cohan (2002) showed that the fundamental properties of species are held, not by the named species of bacterial systematics, but by ecotypes. An ecotype is defined as (Cohan, 2001) a set of strains which use the same ecological niche, such that an adaptive mutant from within the ecotype outcompetes to extinction all other strains of the same ecotype (within the same monophyletic clade), but not the strains from other ecotypes. So, the periodic selection which purges diversity, functions as a force of cohesion, like sexual reproduction (recombination) does in sexual species. The different ecotypes are irreversibly separated, so that each ecotype functions as a fundamental unit of ecology and evolution, like in the *Biological Species Concept*. And asexual ecotypes are, like sexual species, ecologically distinct. Moreover, a speciation event would imply the divergence between ecotypes to become sufficiently strong that they have independent evolutionary futures, so that each ecotype (monophyletic clade) undergoes independent selective sweeps which purge it of variation without displacing the other ecotype. Using this advanced species and speciation definition, the monophyletic clades L and S, discussed in the above experiment, should be seen as distinct species specialized to different ecological niches. Therefore, this implies that during the long-term evolution experiment with *E. coli* a speciation event happened around 6,000 generations in Ara^{-2} that resulted in two monophyletic clades that are sufficiently differentiated to undergo independent adaptive evolution without displacing each other (Rozen *et al.*, 2005). Moreover, evolutionary adaptation quite rapidly made use of the vacant niches, which suggests that adaptive radiation would be a quite common process (without many constrains), as can be expected when looking at the biodiversity in nature. In general, it is important to realize that biological diversification is not bound by our definitions, but simply uses the ecological opportunities that are available (if not constrained by, for example, evolutionary history).

Processes underlying the origin and maintenance of diversity

All the discussed experimental studies on adaptive radiation show the two necessary ingredients for the origin and maintenance of diversity, which are the ecological opportunity and specific selection pressures. Of course, neutral variation is also present within these experiments, but the role of neutral variation in adaptive evolution and diversification will be discussed later. Already in the introduction of this paragraph we discussed the role of ecological opportunity with the *niche exclusion principle*, which simple means that no two species can occupy the same niche. In the later section, the potential role of spatial and temporal heterogeneity in niche specialization and diversification was discussed. Within spatially structured environments, it was clearly shown that when heterogeneity was removed, diversity was quite rapidly lost (Kerr *et al.*, 2002; Rainey & Travisano, 1998). Under the seasonal conditions, resource partitioning by *cross-feeding* played an important role in the maintenance of polymorphism, Turner and colleagues (1996) showed that when the glucose concentration in the environment was lowered, and likewise the excreted metabolites, the lost opportunity for *cross-feeding* resulted in a lowered diversity. So, an ecological opportunity in the form of a vacant niche was needed before any diversification could occur. However the way in which a niche is defined is mainly a theoretical construct which is empirically overwhelming (including all environmental conditions). That diversity was quickly lost when deliberately reducing environmental complexity does not mean that environmental complexity always automatically leads to biological diversity. Buckling and colleagues (2007) showed that under different scales of temporal variation between high and low quality environment, so with the opportunity for niche specialization, an equally well performing generalist could evolve (using *P. fluorescens*). This generalist, in contrast to the cultures that evolved without temporal fluctuations, could perform equally well in both environments. So, the occurrence of diversification also depends on the relative benefits of specialization *vs.* general adaptation. If a species niche range could be extended without additional costs you would expect a generalist to evolve. In other words, not only the environmental opportunity, but also the selective benefit for specialization is needed. In addition, after niche specialists have evolved, selective pressures are needed for the maintenance of diversity.

In general, four different selective regimes could be responsible for the maintenance of genetic variation (Rainey *et al.*, 2000): (i) Heterosis, in which the heterozygote has a selective advantage (this selective

regime does not apply for haploid organisms, like bacteria); (ii) negative frequency-dependent selection, where the fitness of a genotypes is a negative function of its frequency; (iii) selection for recurrent beneficial mutations, where polymorphism is maintained by continual selective sweeps (dynamics should correspond to *Fisher's fundamental theorem* (1930), where the instantaneous rate of change in the mean fitness of a population is equal to the genetic variance for fitness within that population) and (iv) selection against recurrent deleterious mutations, which gives rise to a mutation-selection balance. From these four selection regimes, only the last three could be responsible for maintenance of diversity in microorganisms. In the above described studies only the presence of frequency-dependent selection could be confirmed (Rainey and Travisano, 1998; Souza *et al*., 1997). Elena and Lenski (1997) tested six of the twelve long-term populations for the presence of each selective regime and discovered a small contribution of recurrent deleterious mutations in one population, but a major contribution of frequency-dependent selection in all six populations. Later, also transient polymorphisms due to recurrent beneficial mutations were discovered in some of the long-term populations (Pelosi, *et al.,* 2006; discussed below, but also in other studies similar transient polymorphisms were observed, Atwood *et al*, 1951). This transient polymorphism was detected in maltose metabolism, this in contrast to the variation in fitness that was measured by Elena and Lenski (1997) (Lenski & Travisano, 1994). It could be that the physiological variation that underlies the variation in fitness is more polymorphic due to recurrent beneficial mutations that exert a similar fitness advantage. Also, frequency-dependent selection invalidates the use of mean fitness relative to the common ancestor. Although the average fitness gain relative to the ancestor is much bigger (Lenski & Travisano, 1994) than the advantage of a clone when being rare, after 20,000 generations the rate (Cooper & Lenski, 2000) of fitness increase relative to the ancestor is decelerated so dramatically that the role of frequency-selection may be the most dominant. In conclusion, frequency-dependent selection most strongly contributed to the maintenance of diversity. For future research it would be interesting to known how the observed phenotypic diversity can be mapped on the underlying genetic diversity. In the next section, an attempt to understand the genotype to phenotype mapping will be discussed in the context of evolutionary adaptation, mainly focussing on the long-term evolution experiment of *E. coli*.

THE MULTIPLE LEVELS OF EVOLUTIONARY ADAPTATION

Although diversification was observed in the long-term experiment, the main objective was to study evolutionary adaptation by natural selection. In this paragraph, we exclusively look at the results on evolutionary adaptation within the long-term evolution experiment (Lenski *et al.*, 1991). The vast extent of studies examined: (i) genomic changes (Papadopoulos *et al*., 1999; Schneider *et al*., 2000), (ii) changes in gene expression (Cooper *et al*., 2003), (iii) protein profiles (Pelosi *et al*., 2006) and (iv) phenotype (Lenski *et al*., 1991; Vasi *et al*., 1994). In this way, these studies comprehend the different levels that are present in the genotype to phenotype mapping. Overall, many parallel changes could be observed between the different levels and replicate populations, which is a hallmark of adaptive evolution (Philippe *et al.,* 2007). Our aim is to comprehend the evolutionary adaptations that are discovered within the first 20,000 generations of this long-term experiment, whereby focussing on the multiple levels of regulation that are involved. First, we will look at changes in phenotype, followed by genomic changes, changes in global regulatory networks and changes in local regulons.

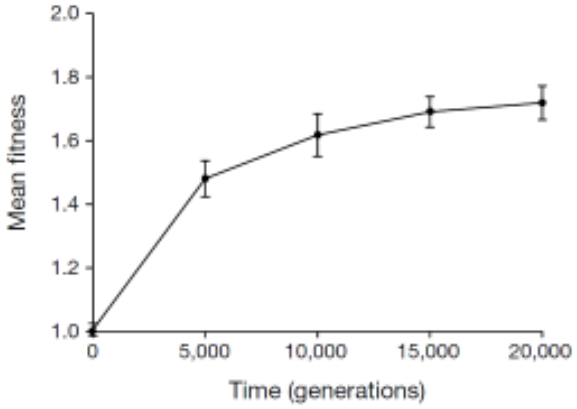


Figure 6. The trajectory for mean fitness of *E. coli* during 20,000 generations in minimal glucose medium (Long-term experiment), for details see Cooper & Lenski (2002). Each point is the mean of all 12 populations, and fitness of each population relative to the ancestor was measured with fivefold replication. Error bars are 95% confidence intervals based on the replicate populations. From Cooper & Lenski (2002).

Phenotypic changes

Changes in fitness and other life-history traits

On average, the fitness gain relative to the ancestral population, after 20,000 generations, was about 70% (Cooper & Lenski, 2000). The increase in fitness decelerated over time, whereby the average rate of relative fitness improvement in the last 5,000 generations was only about one tenth of the average rate in the first 5,000 generations (figure 6; Cooper & Lenski, 2000). There was also a significant amount of between-population variation in this fitness improvement, which may reflect the different adaptive avenues that are taken on the adaptive landscape (figure 10c and 10d) Lenski *et al*., 1991; Lenski & Travisano, 1994). In addition, the changes in

Table 1. IS mediated mutations that are substituted in one of the focal populations and the presence of parallelism in the other replicate populations. The mutational events are characterized by Schneider *et al* . (2000), while the Woods *et al*. (2006) sequenced four of these genes in the other 11 replicate long-term populations to determine the amount of parallelism. So, "other populations" show the number of populations that also had a mutation at the candidate gene. " - " does mean that no test is performed, " * " means that this mutation is tested for parallelism. Adapted from Schneider *et al*. (2000) and Woods *et al*. (2006).

Mutational event	Focal population	Other populations
Insertion of IS*150* in *pykF**	Ara^{-1}	11
Inversion involving existing IS*1* elements and located in *gatZ* and between *citC* and *dpiB*	Ara^{-1}	-
Deletion from IS*1* located in *nmpC* to *E. coli* B-specific sequence	Ara^{-1}	-
Insertion of IS*150* into *nadR**	Ara^{+1}	11
Insertion of IS*150* into *hokB-sokB**	Ara^{+1}	5
Insertion involving new IS*150* element in *nadR* and existing IS*150* in *hokX-sokX*	Ara^{+1}	-
Insertion of IS*150* upstream of *pbpA-rodA* operon*	Ara^{+1}	5
Insertion of IS*150* into *yfcU*	Ara^{+1}	-
Insertion of IS*150* into intergenic region between *glcB* and *yghK*	Ara^{+1}	-

demographic parameters and life-history traits (population density, maximal growth rate (V_{max}), resource concentration at half the maximum growth rate (K_s), duration of the lag phase, death rate during stationary phase, death rate during prolonged starvation, numerical yield in pure culture and average cell size in both stationary and exponential phase) were measured by Vasi and colleagues (1994). A significant increase (~15%) in growth rate and a decrease (~20%) in lag phase were measured over the first 2,000 generations, promoting the exploitation of resources when they were abundant. In contrast, there were no relevant changes in characteristics that facilitate survival when the resources were depleted. So, the bacteria adapted to the 'feast' and not to the 'famine'. Maybe, the ancestor was already well adapted to the 'famine' and therefore lost the opportunity for selection in this period. In addition to these changes, also cell size increased over the first 2,000 generations. This increase could not merely be explained by the allometric correlation between cell size and growth rate (Lenski *et al.*, 1998). It could be that the increased cell size has something to do with changes in cell composition, for instance the ratio RNA to DNA appears to have increased in the derived cells (while this was not the case for the ratio protein to RNA; Lenski *et al.*, 1998). Another consequence of continuous adaptation on a similar substrate is the occurrence of metabolic trade-offs that would result in a reduced fitness in unfamiliar environments, a phenomenon better known as *ecological specialization*.

Ecological specialization

The first ones to study *ecological specialization* in the long-term experiment were Travisano and colleagues (1995), who compared the growth performance of the derived populations on both glucose and maltose. Where the mean fitness increase in glucose was large and little between-population variation existed, there was no average fitness increase in maltose with a significant amount of between-population variation. So, the low variation in the selective environment masks a much greater heterogeneity in their adaptation to other environments (like maltose). Because maltose and glucose metabolism only differs in their mode of transport, the fitness increase in glucose implies that adaptation was primarily achieved by improving glucose transport (Travisano *et al*., 1995). In addition, Dykhuizen and Dean (1990) already had shown that the relative fitness of *E. coli* strains propagated on lactose correlated with metabolic flux. To test if glucose transport was indeed the target of selection, populations were grown on multiple different substrates that varied in their transport system (Travisano & Lenski, 1996). These nutrients were classified by the mechanisms of their transport through the outer (OmpF vs. LamB) and inner (PTS [= phosphotransferase system] vs. non-PTS) membranes, in which glucose was a OmpF / PTS nutrient (maltose is a LamB / non-PTS nutrient). The parallel response of the derived genotypes on all OmpF / PTS nutrients, which was different from the responses on the other nutrients, indicated that both OmpF and PTS were important targets of selection (Travisano & Lenski, 1996). The twelve populations could also be subdivided in six distinct groups when looking at the fitness responses to the novel nutrients. So again, high heterogeneity under the novel environmental conditions was observed.

To understand the underlying genetic mechanism responsible for *ecological specialization* Cooper and Lenski (2000) tested the decay of unused catabolic functions in all twelve populations. In total 95 different carbon sources were used, of which 64 substrates were informative and 16 showed parallel decay in the first 10,000 generations. On average the total catabolic function declined by at least 32%. Two

distinct population genetic processes could be responsible for the observed *ecological specialization*: (i) *mutational accumulation*, where mutations become fixed by genetic drift and are neutral in the selective environment but deleterious in another environment, and (ii) *antagonistic pleiotropy*, a trade-off in which the same mutations that are beneficial in the selective environment are deleterious in another environment. The observation of 16 parallel changes in functional decay implies that *antagonistic pleiotropy* contributes the most to the trade-off observed under *ecological specialization.* In addition, mutator genotypes did not accumulate more functional losses than the other genotypes, which is expected if *mutational accumulation* does not, or only marginally, contribute to *ecological specialization*.

So, together, it was shown that adaptation to a specific environment resulted in *ecological specialization*; this specialization (thanks to the pleiotropic effects of fixed beneficial mutations) resulted in an increased fitness variation under novel environmental conditions. This variation can function as a *latent selection potential* (Travisano *et al.*, 1995). So, although *ecological specialization* on average results in reduced fitness within novel environments, it also causes the accumulation of variation that, in accordance with Fisher's fundamental theorem (1930), accelerates the rate of fitness increase when populations enter the novel environment. Normally this *latent selection potential* is associated with the accumulation of neutral alleles (Kimura, 1983). However, here, pleiotropic effects of fixed beneficial mutations seem to give similar results. But, it should be noted that Fisher's fundamental theorem is based on within-population variation, while the variation described above is between-population. It is questionable that within-population variation can be established as easily as between-population variation, because clonal interference and selective sweeps counteract the accumulation of variation (Gerrish & Lenski, 1998).

Genomic changes

Ultimately, all phenotypic changes, like the ones discussed above, must have a genetic basis to be relevant for adaptive evolution. But before we follow a *top-down* approach for examining the phenotypic changes and their corresponding mutations we shortly discuss the amount of genetic parallelism present among the different populations (the dynamics of genomic evolution will be discussed in more detail in the next paragraph).

To estimate the amount of parallel evolution, *blind candidate* genes are needed. These genes are found by looking for mutations that are substituted in one of the populations without investigation based on parallel phenotypic changes related to their function. By sequencing the same genes in the other replicate population, the amount of genetic parallelism could be measured. One way to find candidate genes without using a *top-down* approach, is by using insertion sequence (IS) elements. IS elements were used as genetic markers to follow changes in genomic diversity, and a very high genetic dynamism was observed in two of the long-term experiments (figure 12; Papadopoulos *et al*., 1999; these dynamics will be discussed in a later section). These IS elements often facilitate mutations by transposition and therefore could be used to find proper candidate genes. Schneider and colleagues (2000) characterized nine IS-mediated mutations in two long-term populations (see table 1). From these mutations, Woods and colleagues (2006) picked four for which the presence of parallelism was tested. So, for these candidate genes all other populations were sequenced and checked for mutations. At the level of mutations little parallelism could be discovered, on average only 2,1% of the mutations were shared between two populations. However, extensive parallelism was present at the level of the candidate genes (see table 1), this in contrast to genes that are chosen randomly (Lenski *et al*., 2003). So, a high degree of genetic parallelism was present, which means that similar genes fixed beneficial mutations over the different independently evolving replicate populations. Because parallelism was observed in blindly chosen candidate genes, the observation of parallelism is not merely a bias of the subset of genes that are included in the analysis, and therefore forms the strongest indication for adaptive evolution. In the next sections we will discuss parallel changes that are discovered using the *top-down* approach (with this approach you only include genes in your analysis that are related with phenotypic characteristics that show parallelism over the different populations), this to better identify the relationship between phenopic and genomic changes.

Global regulatory networks

DNA topology

In a serial transfer regime, there are continuous transitions between exponential growth and starvation. Such transitions are known to influence DNA topology (Hatfield & Benham, 2002). Moreover, phenotypic acclimatisation to the continually changing conditions requires rapid changes in the expression of many genes. Transient modification in DNA supercoiling, which can produce genome-wide changes in gene expression, can contribute to these acclimatization processes. Hence, it is interesting to see if DNA supercoiling changed in the evolved long-term populations (Crozat *et al*., 2005). To measure this trait, a reporter plasmid was introduced in three clones isolated from each population at three different time points (2,000, 10,000 and 20,000 generations). In most of the evolved populations the level of DNA supercoiling significantly increased over the generations. These changes in DNA topology were parallel and often already visible within the first 2,000

generations which indicates adaptive evolution. To identify the underlying genetic changes, candidate genes were sequenced in the Ara^{-1} population. In general, two types of enzymes control the DNA supercoiling of the chromosome: topoisomerase and histone-like proteins (Philippe *et al*., 2007). In Ara^{-1} populations, mutations were found in *topA*, encoding topoisomerase I, and in *fis*, a gene encoding a histone-like protein (the Fis protein). To evaluate the fitness effects of these mutated genes, isogenic strains were constructed by moving the evolved *topA* and *fis* alleles, alone or in combination, into the ancestral background. In combination, these mutations resulted in a fitness advantage of ~15,5% relative to the ancestor. Thus, the increased DNA supercoiling clearly was beneficial for the evolved Ara^{-1} population. In addition, the transitions in DNA supercoiling in the evolved population over time, corresponded with the increase in supercoiling that was experienced by the isogenic strains. So, *topA* mutant was substituted by 2000 generations, and induced ~12% change, while the *fis* mutant was substituted later and induced an additional ~5% change in DNA supercoiling (Crozat *et al*., 2005).

The regulatory interactions involved in adjusting DNA supercoiling are quite complex. Most likely, the *topA* mutant decreases the activity of topoisomerase I, which results in an increase in DNA supercoiling. The Fis protein, encoded by the *fis* gene, normally represses *gyrAB* genes, which encode DNA gyrase, the only enzyme able to introduce negative supercoils into DNA molecules (Schneider *et al*., 1999). So, the *fis* mutant probably increases DNA supercoiling by reducing its repression of *gyrAB* genes. An increase in DNA supercoiling would increase the transcription of the rRNA operons (Schneider *et al*., 1999). A higher rate of rRNA synthesis would be beneficial in the evolved population by facilitating higher exponential growth rates. As described above, Lenski and colleagues (1998) also observed an increased RNA:DNA-ratio in the evolved populations.

The relative slow substitution rate of *topA*, when considering his >10% fitness advantage, suggested clonal interference with other equally beneficial mutations (Gerrish & Lenski, 1998). And indeed, when looking at the focal population at generation 1,000, other clones were found with a similar increase in DNA supercoiling and fitness, but without the *topA* mutation. These parallel changes indicates that DNA topology was a key target of selection. For future research it would be interesting to know if the other populations substituted similar mutations and how exactly these mutations change the rRNA level.

Stringent response

Also at the level of gene expression, parallelism was examined (Cooper *et al*., 2003). Microarray analysis was performed on all 4,290 genes, to compare the gene expression patterns of the derived populations, Ara^{+1} and Ara^{-1}, with that of their ancestor. Like expected in convergent adaptive evolution, the derived populations were more similar to each other than they were to their ancestor. To examine which genes were important for adaptive evolution, genes were identified that had significant expression changes relative to their ancestor, in both populations. In total, 59 genes fulfilled this stringent criterion, and remarkably, all 59 genes changed in parallel. This extreme parallelism in two independently evolving populations was a strong indication for adaptive evolution. However, it does not mean that all these changes were reflecting separate mutations. In contrast, mutations in global regulatory networks were far more likely. And, indeed, more than half of these parallel-responding genes were regulated by specific effectors including guanosine tetraphosphate (ppGpp) and cAMP-cAMP receptor protein (CRP) (Cooper *et al*., 2003). The cellular concentration of ppGpp itself is affected by the products of two genes, namely *relA* and *spoT* (Cashel *et al*., 1996). The concentration of cAMP-CRP is dependent on the products of *cyaA* and *crp*, which in turn are also influenced by ppGpp (Johansson *et al*., 2000). So, therefore these four genes were interesting candidates for harbouring beneficial mutations. However, from all these genes only *spoT* was shown to have a mutation in the Ara^{-1} population, in which a lysine was replaced by an isoleucine. When an isogenic strain was constructed by moving the evolved *spoT* allele into the ancestral background, a fitness gain of ~9,4% could be measured. In addition, the changes in gene expression from 11 of the 59 parallel-responding genes were also present in the isogenic strain, so the *spoT* mutation was by itself responsible for a substantial amount of expression changes that were observed after 20,000 generations. When also the other replicate populations were examined for potential mutations in *spoT*, they found that 8 of the 12 populations had non-synonymous mutations in their *spoT* gene. Thus, by using parallel changes in gene expression, beneficial mutations could quite easily be detected.

A similar *top-down* approach was applied with the usage of global protein profiles (Pelosi *et al*., 2006). These profiles were made by two-dimensional protein electrophoresis, with proteins extracted from cells harvested after the stationary phase in a 10-fold higher concentrated glucose medium to obtain more proteins (this in contrast to the microarray analysis that used cells from the exponential growth phase under the normal selective conditions). From the around 400 genes that were examined, 38 showed parallel changes in their expression relative to the ancestor in both Ara^{+1} and Ara^{-1}. Pelosi and colleagues (2006) described four aspects in which the proteomic data supported the transcription profiling: first, several dozen genes exhibit altered gene expression in both independently evolved long-term populations; second, with both approaches all changes that affected both populations were parallel, which suggests physiologically relevant events in adaptive evolution;

third, most of these parallel changes involved decreased expression; and last, both approaches implicate that the parallel changes were in the same functional categories of genes. Moreover, when the proteomic data were examined in greater detail an even more striking similarity was observed, namely half the proteins found with protein profiling were regulated by ppGpp and cAMP-CRP. So, the parallel changes observed after 20,000 generations of experimental evolution using mRNA and protein profiling methodologies reflected the same underlying basis of adaptation, which is the previously described *spoT* mutation.

The *spoT* gene is part of the global regulatory network called the *stringent response*. This network is involved in the physiological adaptation to nutritional stress (Cashel *et al*., 1996). More precisely, in starvation *spoT* encodes a protein involved in the synthesis of the alarmone ppGpp, then ppGpp reprograms the entire transcriptional machinery of the cell. As a consequence, the synthesis of stable RNAs is strongly inhibited along with various other processes that are involved in cell growth. Ultimately, ppGpp stimulates the transcription of genes that promote survival during starvation, like the alternative sigma factor RpoS (master regulator of the *general stress response*). Cooper and colleagues (2003) argued that the *spoT* mutation could result in a decreased level of ppGpp, that ultimately increases the rate of transcription from tRNA and rRNA promoters. This, subsequently, would result in an increased maximal growth rate due to an increased speed of translation. However, there were no measurable differences in the dynamics or overall level of ppGpp between the isogenic strains bearing the ancestral and evolved *spoT* alleles (Philippe *et al*., 2007). In contrast, Cooper and colleagues (2003) found that 62 of the 94 tRNA and rRNA-associated genes showed increased expression under the isogenic strain bearing the evolved *spoT* allele (the microarray analysis only involved mRNA, so therefore this indirect way of detecting the levels of tRNA and rRNA). In addition, the rate of tRNA and rRNA synthesis is strongly correlated with growth rate and declines sharply during the transition into the stationary phase (Bremer & Dennis, 1996). In these transitions, the genes *spoT*, *topA* and *fis* are known to be important (Philippe *et al*., 2007). In consequence, Philippe and colleagues (2007) suggested that the evolved cells are poised in stationary phase to respond more quickly to the nutrients that are predictably supplied each day, because not only an increased maximal growth rate was observed, but also a proportionally stronger decrease in lag phase. In this way, the transitions between 'feast' and 'famine' as a consequence of the daily transfer regime, did not merely result in an increased adaptation to the periods of 'feast', but also to an advanced global regulatory network that responded more quickly to the bi-directional transitions.

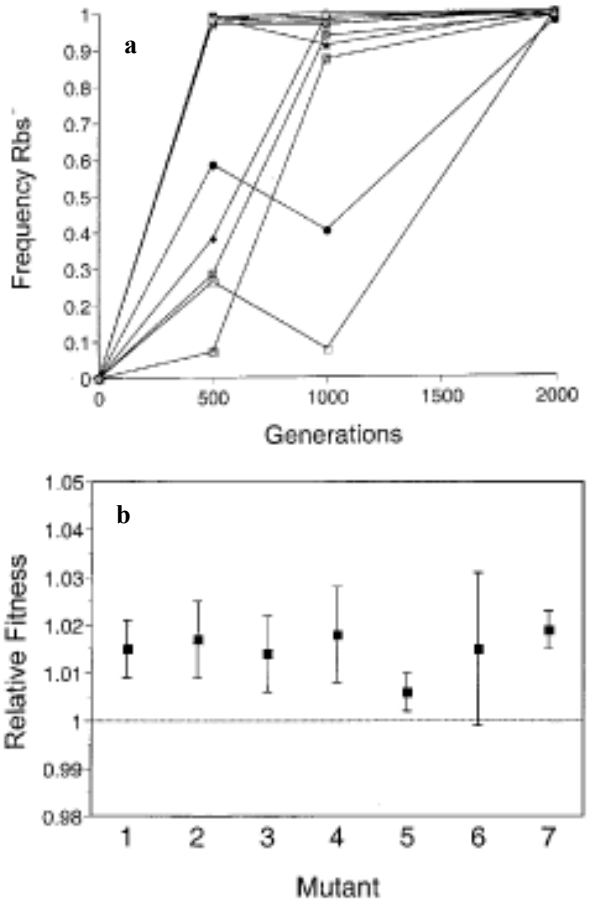


Figure 7. Frequency changes (**a**) and fitness advantages (**b**) of the Rbs⁻ mutants, that lost their ribose catabolic function, see for details Cooper *et al*. (2001). **a**, frequency of Rbs⁻ cells over time in the 12 evolving populations. At generation 2000 all the populations contained between 97 and 100% Rbs⁻ cells. **b**, Fitnesses of seven spontaneous Rbs⁻ mutants relative to their progenitor, as measured by competition experiments. Error bars show 95% confidence intervals based on fivefold replication for each mutant. Adapted from Cooper *et al*. (2001).

In conclusion, the 20,000 generations of experimental evolution under the serial transfer regime, has reshaped and improved the interplay between the DNA topology and *stringent response* networks (Philippe *et al*., 2007). To better understand adaptive evolution, we evidently have to understand the consequences of 'local' changes in these highly complex networks (Barabasi & Oltvai, 2004). So, although adaptive evolution is the consequence of mathematical processes that happen automatically (survival of the fittest), if we want to understand it, we need to comprehend its underlying complexity and the selective advantages of genes that are being substituted.

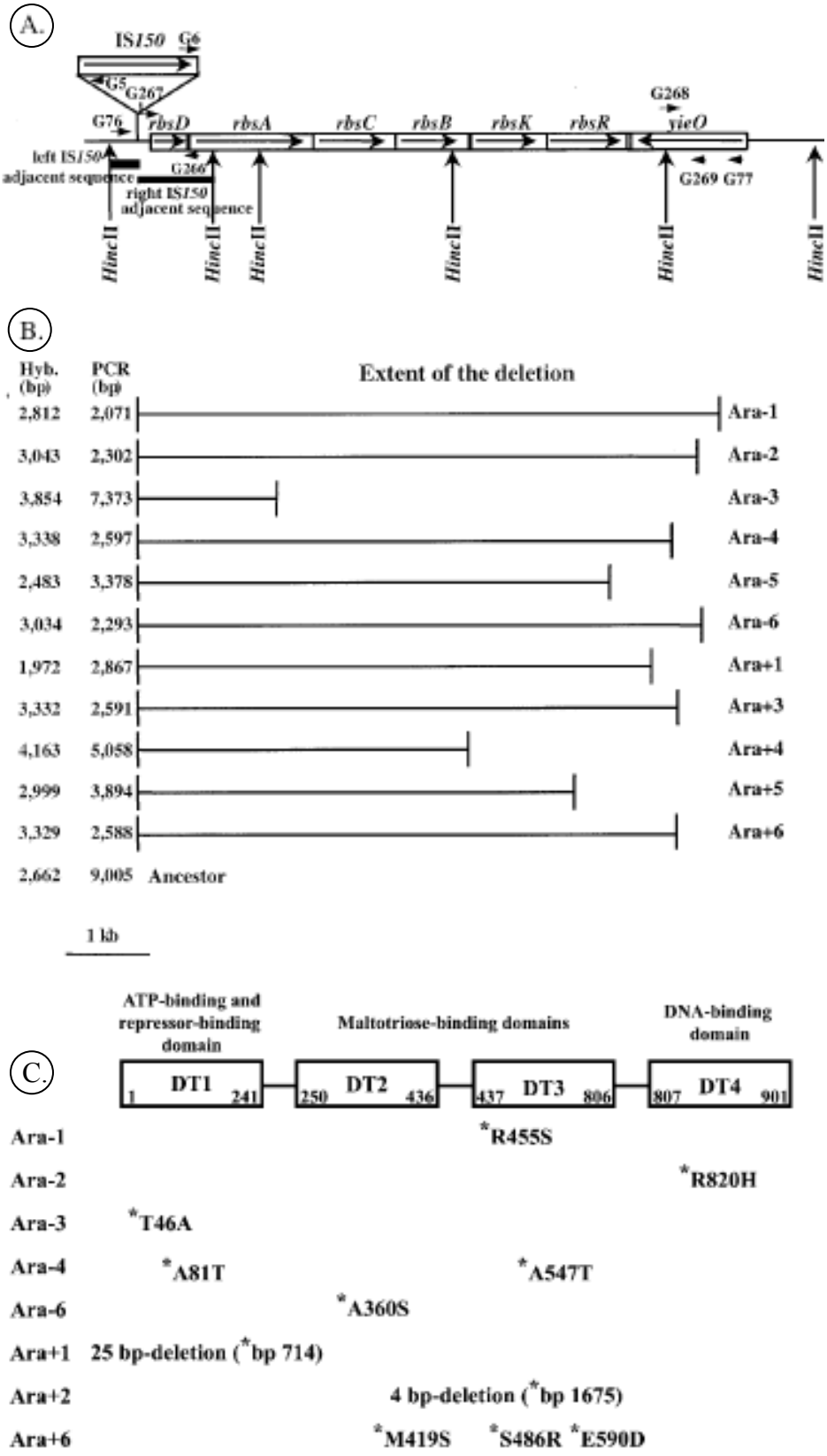


Figure 8. Mutations that cause the loss of ribose catabolism (**a** & **b**) and maltose catabolism (**c**), see for details Cooper *et al* (2001) and Pelosi *et al*. (2006). **a**, Map of the *rbs* operon in *E. coli* B ancestor, based on the genome sequence of *E. coli* K12 (Blattner *et al*., 1997). For details see Cooper *et al*. (2001). Arrows show the direction of gene transcription. An IS*150* element is located upstream of the *rbs* operon. **b**, The deletions (in *rbs* operon) in clones isolated from 11 of the 12 poopulations are shown as horizontal lines (only Ara^{+2} is not depicted). Size of sequence hybridization shown by the label Hyb. (bp). Likewise the sizes of PCR product are shown by the label PCR (bp). As indication for the size of the deletion, at the bottom the reference sequence length of the ancestor is shown. **c**. location of the mutations that cause a decreased expression of the MalT protein or a total loss of this expression. Above the four domains in this protein are shown. The *MalT* gene was sequenced in one clone isolated at 20,000 generations from each of the twelve populations (only the populations with mutations are shown). " * " indicate the presence of an amino-acid substitutions, only the Ara^{+1} and Ara^{+2} long-term populations have small deletions. Adapted from Cooper *et al*. (2001) and Pelosi *et al*. (2006).

Local regulons

The ribose operon and maltose regulon

Besides the complex changes in global regulatory networks, like DNA topology and the *stringent response*, proteomic and transcriptomic studies also revealed parallel changes in two smaller regulons: the ribose operon and maltose regulon. Ribose catabolism was lost in all twelve long-term populations after 2,000 generations. As expected, the loss of this costly

catabolic function resulted in a fitness increase of about 1 to 2% (figure 7a and 7b; Cooper *et al*., 2001). However, this fitness increase was small relative to the rapidity with which all populations lost their ribose catabolic function, for example, the *topA* mutant, that gave a >10% fitness advantage, was also substituted after 2,000 generations. The rapid loss could be explained by an unusually high mutation rate from Rbs^+ to Rbs^-, which was mediated by an IS*150* element. The hypermutable genetic basis mainly resulted in deletions of the *rbs* operon adjacent to the IS*150* element (figure 8a and 8b).

Similarly, a reduced activity of the maltose regulon was observed in 8 of the 12 populations (Pelosi *et al.*, 2006). Parallel substitutions were found in *malT*, the transcriptional activator of the maltose regulon. The affected domains were variable, as well as the mutation types, which included deletions and amino acid substitutions (figure 8c). By using isogenic strains in which evolved *malT* alleles were moved into the ancestral background, it was shown that most *malT* mutation resulted in an absence of growth on maltose and the loss of LamB. LamB is an outer-membrane maltoporin, which is encoded by *lamB*, one of the targets of the MalT transcriptional activator. The *malT* mutation resulted in a fitness gain of about 1% under the selective conditions of the long-term experiment, by preventing the costs of maltose catabolism. Another very interesting aspect of the observed loss in maltose catabolic function is the high level of transient diversity that was observed, with at least three phenotypic and five genotypic subpopulations present between generations 3,000 and 5,000 in the Ara^{+1} population. Like explained in the previous paragraph, four selection regimes could be responsible for the maintenance of diversity. One of them was the selection for recurrent beneficial mutations, which is also observed here. The three phenotypic groups are (i) Mal^+ which has the ancestor phenotype (growth after 8 hrs), (ii) Mal^s which has a reduced growth on maltose (growth on maltose after 24 hrs) and last (iii) Mal^- which lost the maltose catabolic function completely (no growth after 24 hrs). So, first Mal^s was selected followed by Mal^-, which had a greater selective advantage (figure 9). Similar transient polymorphism are also observed in other populations.

In conclusion, the parallel losses of unused and costly catabolic functions is a strong indication for adaptive evolution. In this way, the long-term evolution experiment in a glucose minimal medium resulted in *ecological specialization*. In addition, it was shown that the same underlying genetic mutations that resulted in the loss of a catabolic function also increased fitness, therefore these examples show *direct* evidence of *antagonistic pleiotropy* as underlying genetic mechanism of *ecological specialization* (discussed above; Cooper & Lenski, 2000). These examples also show that different types of mutations can underlie similar changes in phenotypic characteristics, which is not hard to imagine if a reduction in catabolic activity is beneficial.

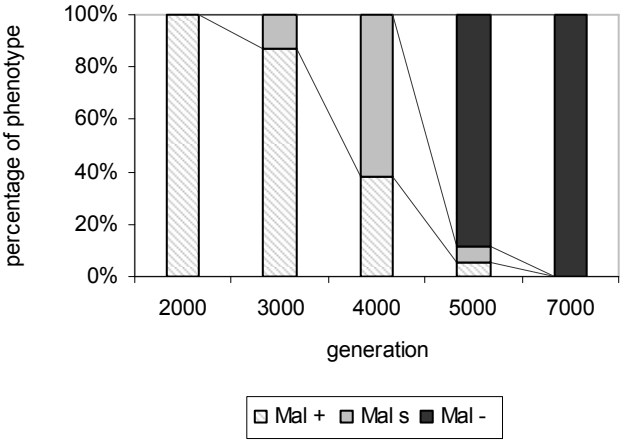


Figure 9. Phenotypic evolutionary dynamics of maltose growth ability. Individual clones were isolated at several time points from population Ara^{+1} and assessed for their growth capacity in DM250 maltose medium at 37 °C. Here, the relative frequencies of the different *phenotypes* are depicted over time. Mal^+ (shown as Mal +, *striped*), growth after 8 hr; Mal^- (shown as Mal -, *black*), no growth after 24 hr; Mal^S (shown as Mal s, *grey*), no growth after 8 hr but growth after 24 hr. Adapted from Pelosi *et al*. (2006).

Conclusions

So, in conclusion, after 20,000 generations of independent experimental evolution the different replicate populations show a widespread parallelism at various levels. At the phenotypic level, all the population show a decelerating fitness increase associated with changes in cell size, growth rate and duration of lag phase, in comparison to their common ancestor (Lenski *et al*., 1991; Vasi *et al*., 1994). All populations also exhibit increased specialization on glucose with associated reductions in other catabolic functions. *Antagonistic pleiotropy* seem to be the most important underlying genetic basis, and is even directly proven to be the cause of *ecological specialization* for two catabolic functions (Cooper & Lenski, 2000; Pelosi *et al*., 2006). Most populations also showed similar changes in global regulatory networks, which was an increased DNA supercoiling (Crozat *et al*., 2005) and changes in the *stringent response* (Philippe *et al*., 2007). These adaptations seem to be involved in the ongoing bi-directional transitions between 'feast' and 'famine' that occurred in the experimental environment thanks to the serial transfer regime.

These parallel changes in phenotype overlie a high level of genetic parallelism. Genetic parallelism was mostly found on the gene level, which means that similar genes fixed beneficial mutations over the different independently evolved populations, but that the specific mutations on nucleotide level could be quite different (Woods *et al*., 2006). Because different

mutations affected the same genes, it would be interesting to know if these mutations exerted their beneficial effects via the same or different molecular mechanism. This is particularly interesting because many target genes (*Top A, fis* and *spoT*) encode global regulatory proteins, and therefore different mutations may have widespread pleiotropic effects. In general the widespread parallelism reflects strong adaptive evolution that has occurred during the long-term evolution experiment.

In addition, adaptation occurred on multiple genetic levels, ranging from the level of regulons up to global regulatory networks. This dichotomy reflects the overall architecture and topology of complex regulatory networks in which most genes have only a few connections to other genes, whereas a few genes interact with many other genes (Barabasi & Oltvai, 2004). An improved understanding of regulatory networks is needed to better asses the connection between genetic changes and phenotypic outcomes. Besides knock-out experiments, experimental evolution can help with that, because in experimental evolution the most integrative and complex phenotype of all, which is the fitness of an organism, is being studied. This fitness is inherently the phenotypic outcome of all the interdependent and multiple layers of regulation, and therefore reflects the physiological state of a cell as a whole.

So finally, until now, a total of five different genes or operons have shown to be targets of selection in the long-term evolution experiment based on competition assays performed with isogenic strains: *rbs* (Cooper *et al*., 2001), *malT* (Pelosi *et al*., 2006), *topA* and *fis* (Crozat *et al*., 2005) and *spoT* (Cooper *et al*., 2003). Assuming that the fitness gains from all these mutations are additive, they as a whole would account for a ~30% fitness increase, which is less than half of the ~70% fitness gain that is measured over the 20,000 generations (Cooper & Lenski, 2000). So, many more beneficial mutations await discovery, which is consistent with the ~10-20 beneficial mutations that are expected to be substituted in each population (Lenski, 2004). All these genes are discovered by using a *top-down* approach, in which parallel phenotypic changes are measured by transcriptomic or proteomic profiling, whereafter target genes are sequenced that could be involved in the observed phenotypic changes. In addition to this approach, Woods and colleagues (2006) found four genes that showed parallel genetic changes over the different populations, by using IS-mediated mutations. However, for these genes no isogenic constructs have been made, so their fitness effects remain unknown.

In the next paragraph we will focus on the evolutionary dynamics observed within the long-term populations; many of these dynamics have already been discussed in the previous paragraphs. However, a comprehensive view will shed light on the fundamental processes that are involved in Darwinian evolution.

EVOLUTIONARY DYNAMICS

Until now, we discussed adaptive radiation and evolutionary adaptation by natural selection, two fundamental aspects in evolution. However, the evolutionary dynamics of these processes is only fragmentally considered. In this paragraph, we therefore solely look at these dynamics and try to answer several questions, like: how important is evolutionary history for future adaptation? what is the role of clonal interference? does genetic variation changes over time? Again, as in the previous paragraphs, most of the considered data come from the long-term evolution experiment. As a consequence, in explaining the dynamics we will often refer to the previous paragraphs. Because of the enormous amount of data, and likewise results, evolutionary dynamics is considered separately. However, it is important to realize that, in the end, the integrative view of diversification, evolutionary adaptation and their dynamics, together, is needed to completely comprehend evolution. These dynamics are best described by looking at the multiple levels of cellular organization. Therefore, we first focus on phenotypic dynamics and then on genotypic dynamics. Of course, both levels are highly interconnected.

Phenotypic evolution

The dynamics of evolutionary adaptation

As described in the beginning of the previous paragraph, the average fitness, relative to the ancestor, increased ~70% over 20,000 generations (Cooper & Lenksi, 2000). However, the rate of increase decelerated with time, in which the rate of fitness improvement over the first 5,000 generations was tenfold greater than in the last 5,000 generations. Although this deceleration, a fitness plateau still was not reached. Multiple factors can contribute to this ongoing adaptation and explain why the genetic potential for adaptation was not exhausted after thousands of generations (Elena & Lenski, 2003). First, the amount of time that is needed before a beneficial mutation becomes fixed is inversely dependent on its selective advantage. Lenski and colleagues (1991) showed that given the population size in the long-term experiment, around 250 generations are theoretically needed for the fixation of a mutation with a 10% fitness advantage, while a mutation with 1% fitness advantage would need around 2500 generations before it becomes fixed. Qualitatively similar results are obtained when looking at the fixed mutations described in the previous paragraph. Namely, the *malT* mutation with the fitness advantage of about 1% was fixed in about 7,000 generations (Pelosi *et al*., 2006), while the *spoT* mutation with a fitness advantage of about 10% was fixed in only 1,500 generations (Cooper *et al*., 2003). However, there are two discrepancies between the theoretical prediction and the experimental results.

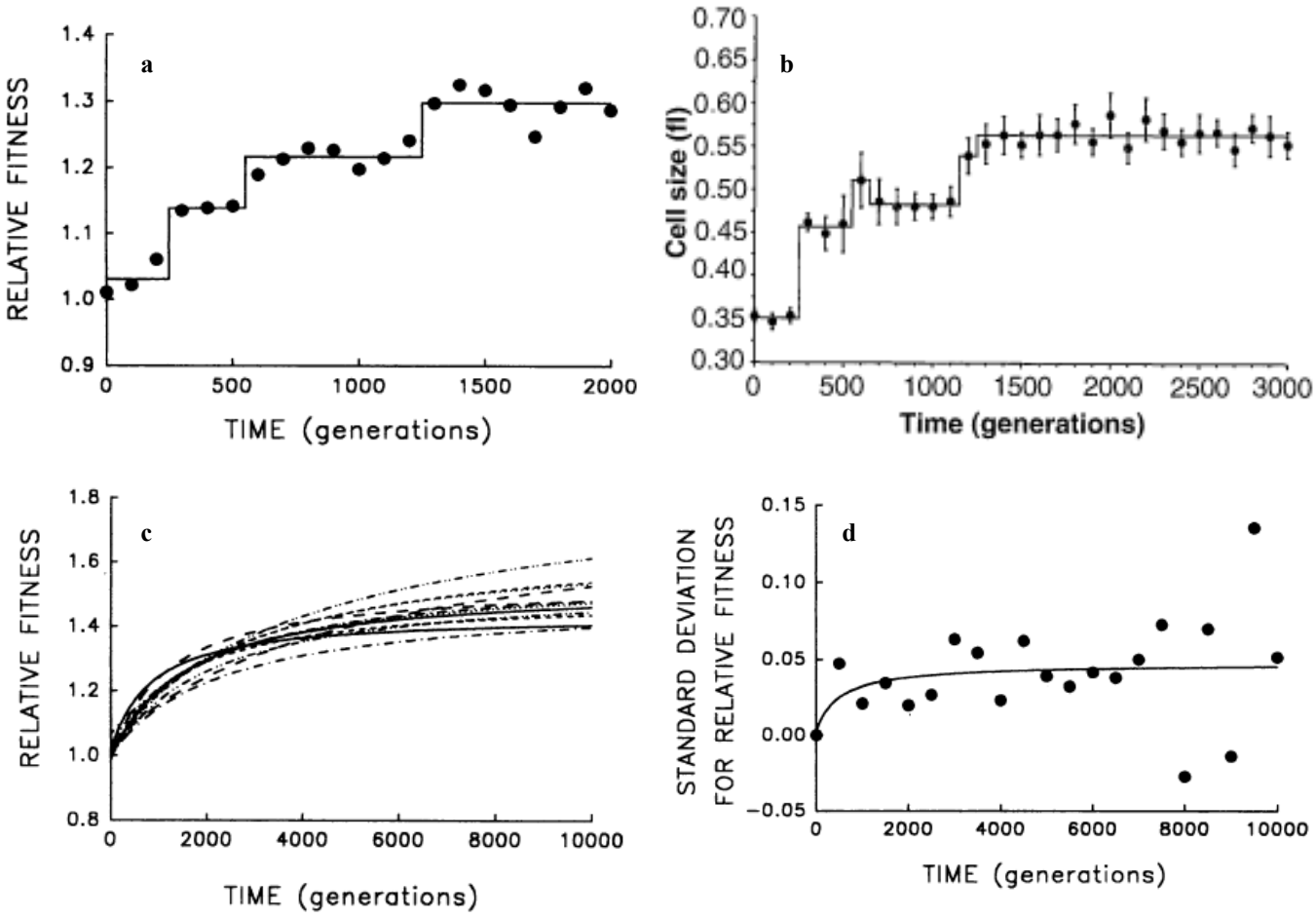


Figure 10. Population dynamics of fitness and cell size of *E. coli* in long-term experiment, see for more details Lenski & Travisano (1994) and Elena *et al*. (1996). **a**, Step-like dynamics in relative fitness improvement, when measuring at frequent intervals (the first 2,000 generations). Solid line indicates the best fit of a step model. **b**, Similar than dynamics in fitness improvement, step-like dynamics in cell-size over the first 3,000 generations. Fitness is shown to be strongly correlated with cell size, see Elena *et al*. (1996). **c**, Trajectories for mean fitness relative to the ancestor in the 12 long-term replicate populations during 10,000 generations. Each curve represents the best fit of a hyperbolic model to data obtained for one population every 500 generations. **d**, Trajectory for the diversification of the 12 populations in their mean fitness. Analyses of variance were performed at 500-generation intervals to partition the observed variation in mean fitness into its components. Each point represents the among-population standard deviation for mean fitness; negative values indicate that the estimated variance component was negative. Curves shows the best fit of a hyperbolic model. Adapted from Lenski & Travisano (1994) and Elena *et al*. (1996).

First, having a similar fitness advantage, the time before a mutation becomes fixed is much longer in the experimental results than theoretically is predicted. And second, the difference in time before a mutation becomes fixed between the *malT* mutation and *spoT* mutation is not tenfold, like theoretically is predicted. These discrepancies could be explained by the other factors that contribute to the ongoing evolutionary adaptation. The second factor that contributes to the ongoing adaptation is random drift. Many beneficial mutations while being rare are lost thanks to random drift. The probability that a beneficial mutation survives extinction by random drift is about twice its selective advantage (Elena & Lenski, 2003; Lenski *et al*., 1991). So, the *spoT* mutation with a fitness advantage of about 10% would need on average five 'tries' before it can be established, while *malT* (1%) would need fifty 'tries'. So, both the time needed to become fixed and the chance to survive extinction by random drift are reversely correlated with a mutation's selective advantage. This implies that adaptation using mutations with progressively smaller benefits can continue indefinitely, although with a decelerating rate of improvement, without depleting the supply of useful variants. A third factor that contributes to the ongoing adaptation is the large amount of mutations conferring small adaptive advantages relatively to the small number of mutations that confer a big fitness advantage (Elena & Lenski, 2003). And the last factor that could contribute is clonal interference (Gerrish & Lenski, 1998). Because beneficial mutations are linked to the genetic background in which they occur, multiple beneficial mutations in different genetic backgrounds will compete with each other, which is called clonal interference. Clonal interference logically is confined to asexual organisms in which no recombination, to decrease the linkage disequilibrium between beneficial mutations and their genetic backgrounds, is possible. The impact of clonal

interference on the evolutionary dynamics will be discussed in the next section.

But before we continue, the way in which fitness was measured during the long-term experiment will briefly be discussed. Like already pointed out in the first paragraph, fitness measurements relative to the ancestor are invalid when non-transitive evolution is involved. Non-transitivity was discovered in multiple long-term populations, in most populations the fitness advantage when being rare was only weak (Elena & Lenski, 1997), however in one population strong frequency-dependent selection (non-transitivity) resulted in a stable coexistence (Rozen & Lenski, 2000). To test if the observed deceleration in the rate of fitness increase was merely the consequence of measuring the fitness relative to the ancestor while the interactions were non-transitive, de Visser and Lenski (2002) made a transitive and non-transitive model which both, equally well, could explain the observed data. By comparing the qualitatively different predictions of these models with experimental data, one could see, if the observed deceleration was indeed the consequence of incorrect fitness measurements. Eventually, they found that the results clearly matched the transitive model in all their theoretical predictions. In addition, no statistical deviation from the transitive model could be found. So, the evolutionary dynamics observed in the long-term experiment perfectly reflects the rate of adaptive evolution and the observed deceleration in the rate of fitness increase was not the consequence of incorrect fitness measurements.

Clonal interference

Gerrish and Lenski (1998) examined the importance of clonal interference for the evolutionary dynamics of asexual populations. The prevalence of clonal interference increases dramatically with the number of beneficial mutations that occur in a population, which is dependent on the product of both population size and mutation rate. In this section, we summarize the most important consequences of clonal interference.

First, the probability that a given beneficial mutation will be fixed, declines with a higher population size or mutation rate, which is the logical consequence of an increasing prevalence of clonal interference under these conditions. Second, substitutions appear as discrete, rare events, no matter how frequently beneficial mutations arise. In other words, the trajectory of mean fitness of any asexual population should be punctuated, with short burst or rapid increase followed by long periods of stasis (regardless of the size of the population or its mutation rate). This looks contradicting to the results that are shown in figure 6, however when measuring the fitness more frequently, step-like dynamics could be observed (figure 10a and 10b; Lenski & Travisano, 1994).

The step-like dynamics reflects beneficial mutations that sweep through the evolving population. Initially these punctuated dynamics, after also being observed in morphological characteristics, were argued to be useful in explaining the punctuated-equilibrium model based on the 'fossil record'(Lenski & Travisano, 1994). However later this relevance has been debated (Elena *et al*., 1996; Gould, 2002). Third, the rate of fitness improvement should show diminishing returns with an increasing mutational supply due to clonal interference. So, the rate of fitness improvement will initially increase with an increasing mutational supply (when it is still low), but eventually there will be a 'speed limit' due to competing beneficial mutations. And indeed, in experimental populations of *E. coli*, the rate of evolutionary adaptation was only proportionally related to mutational supply when it was small (de Visser *et al*., 1999). With an increasing mutational supply the amount of competing beneficial mutations also increases, resulting in a 'speed limit', this was also observed in the experiment. In addition, they showed that this 'speed limit' was not present when using already well-adapted populations, because in these populations the degree of beneficial mutations was much lower and likewise the degree of competition between them. So, in general, clonal interference imposes a speed limit on adaptive evolution and the rate of adaptive evolution is therefore not always limited by the mutational supply. Fourth, the rate in which a beneficial mutation spreads through the population should be slower than otherwise predicted from its fitness advantage. Also this theoretical prediction was confirmed by experimental data. Namely, the *topA* mutation discussed in the previous paragraph, had a fitness advantage of more than 10% and is therefore expected to reach a 100% frequency in less than <500 generations (considering the time needed to become fixed and the chance to survive extinction by random drift; Lenski *et al*., 1991). However, the *topA* mutation was only substituted after 2000 generations, this because of competition with a subpopulation that contained another beneficial mutation that resulted in a similarly improved phenotype (~12% increase in DNA supercoiling). Five, clonal interference also results in many beneficial mutations that only transiently become common and thereafter disappear. This was shown to be the case with the Mal^s subpopulation that is discussed in the previous paragraph (figure 9). This transiently common subpopulation had a beneficial point-mutation in the *malT* gene that resulted in a decreased growth on maltose. However, eventually this advanced subpopulation was replaced by another subpopulation that totally lost its maltose catabolic function (Pelosi *et al*., 2006). These results therefore indicate that asexual evolving populations do not necessarily experience discrete selective sweeps, as is supposed under the classical 'periodic selection' model (Atwood *et al*., 1951). But instead, contain many subpopulations, each with different beneficial mutations, which can coexist for long periods even as

they compete with each other. In some cases, transient polymorphism may even give rise to a 'leapfrog' event, in which the majority genotype at some point in time is less closely related to the immediately preceding majority genotype than to an earlier genotype (Gerrish & Lenski, 1998). Papadopoulos and colleagues (1999) observed such a 'leapfrog' event. Namely, the dominant clade (70%) in the Ara^{+1} long-term population at generation 5,000 was no longer seen at generation 8,000, which means that the dominant clade at generation 8,000 emerged from outside the earlier majority type (figure 12; Papadopoulos, 1999).

So in general, clonal interference in combination with the processes described in the previous section, cause adaptive evolution to occur through the substitution of relative few mutations that confer large benefits, as opposed to countless mutations with small benefits (Elena & Lenski, 2002; Rozen *et al*., 2003). This was already indicated in the beginning of this paper, were the number of substituted mutations was calculated to be between 10 and 20 (Lenski, 2004). These mutations together are responsible for the ~70% fitness increase observed after 20,000 generations of experimental evolution (Cooper & Lenski, 2000).

The dynamics of diversification and the adaptive landscape

In the first paragraph we discussed diversification by adaptive radiation, in which natural selection drives populations to diversify in order to occupy vacant niches. The two main ingredients needed were characterized as ecological opportunity and the proper selection regime. The ecological opportunity could be seen as the vacant niche and the selection regime was both responsible for the origin and the maintenance of phenotypic diversity. However, natural selection is not the only fundamental process that contributes to evolution, like discussed in the introduction, random mutations and genetic drift (mixis was not applicable for the organisms considered in this review) also play a role. Random mutation and genetic drift, like discussed in the first section of this paragraph, can be seen as chance effects; like the chance that a particular beneficial mutation survives extinction by genetic drift. Chance events responsible for the diversification of phenotypic characteristics are often considered to be selectively neutral. In addition, the order in which mutations are substituted is often unique and can either constrain or facilitate the substitution of new mutations. In other words, evolutionary history can play an important role in future adaptation. Of course, evolutionary history itself is the product of chance and selection events from the past. So, in general, the diversity that is observed among organisms is the product of three fundamental evolutionary influences: adaptation (natural selection), chance (random mutations and genetic drift) and evolutionary history (selection or chance events from the past that either constrain or promote particular evolutionary outcomes).

Travisano and colleagues (1995) performed an experiment to separate the relative contributions of adaptation, chance and evolutionary history to the phenotypic evolution of both cell size and fitness. They showed that the phenotypic evolution of fitness was highly parallel and convergent among the different replicate populations, in which adaptation almost entirely eliminated the initial 'historical' differences. Also, there was no significant diversification due to chance events. Thus for the phenotypic evolution of fitness, natural selection clearly was the dominant evolutionary process. In contrast, for cell size, the contribution of chance and evolutionary history were more significant. In conclusion they argued that the results supported a general view. Namely, that the effect of history obliterates for traits that are subject to strong selection while is preserved for traits that are subject to weak selection. However, they did not mention the hierarchical organization of these phenotypic characteristics, in which the fitness is the ultimate and most integrative phenotype. In general, one would expect two levels of neutrality and robustness to exist. Namely in the genotype to phenotype mapping and in the phenotype to fitness mapping. Fitness is therefore the complex outcome of the interaction between the phenotype and its environment. In this way, the conclusion could (also) be that evolutionary history does not affect the complex outcome of this interaction, but could affect the different components that contribute to it. However, this experiment was performed in a relative short time period in which the contribution of chance events may be still minor.

Indeed, in the long-term experiment, although initiated from the same ancestor and evolved in the same environment, the replicate populations significantly diverged from each other in both cell size and fitness (figure 10c and 10d; Lenski & Travisano, 1994). Because all population were subject to the same selection regime, the observed diversification demonstrates the importance of chance events in adaptive evolution. However, the observed diversification could still be the consequence of the serial transfer regime, in which populations are continuously forced to go through bottlenecks. These bottlenecks would reduce the variation and likewise increasing the influence of chance events (although the 'bottleneck' population size was still quite big). Finkel and Kolter (1999) prevented the loss of genetic diversity by using constant batch cultures in which no cells were removed. They started the experiment with two isogenic populations which subsequently evolved in a similar environment for only 30 days. After this period both populations were forced to compete with the same competitor. The observed dynamics in competition with this same competitor, differed strongly between the two populations, which indicates that by chance different mutations were substituted in

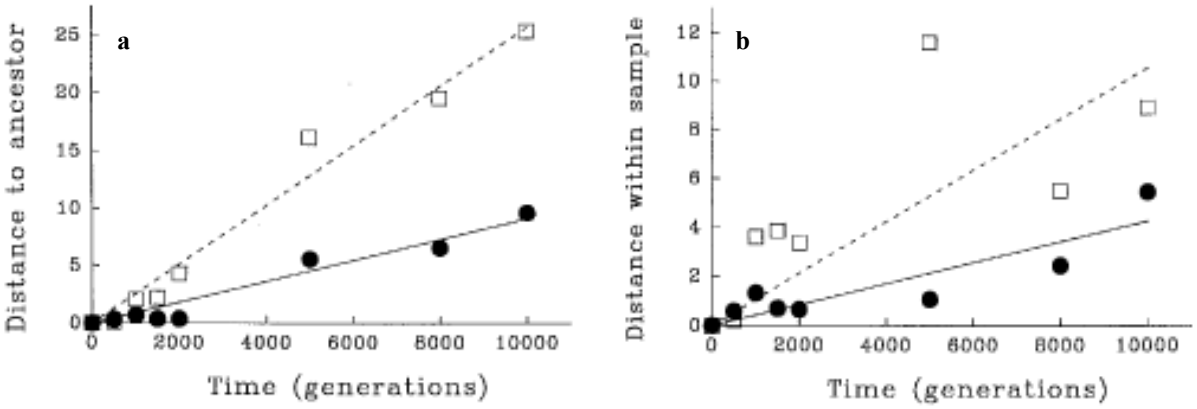


Figure 11. Dynamics of genomic evolution using RFLP-IS analysis, for two long-term populations ([Ara^{+1}] and [Ara^{-1}]) during the first 10,000 generations, for more details see Papadopoulos *et al*. (1999). **a**, Trajectory of average genetic distance between the evolving populations and their common ancestor, calculated as the number of genomic changes detected using IS elements as probes. Solid circles, solid line: Ara^{-1} population. Open squares, dashed line: Ara^{+1} population. **b**, Trajectory of the average genetic diversity within two evolving populations, as calculated from all pairwise genetic distances. Solid circles, solid line: Ara^{-1} population. Open squares, dashed line: Ara^{+1} population. From Papadopoulos *et al*. (1999).

both populations. These results are comparable to *ecological specialization* (discussed above), where the effects of different mutations that confer similar phenotypic benefits in the selective environment, vary in a novel environment (Travisano *et al*., 1995). So overall, these results show that natural selection will cause historical differences to diminish but can not prevent the accumulation of between-population variation in fitness. In addition, the diversity in fitness overlies a much greater heterogeneity of mutational substitutions and physiological characteristics.

The diversification in fitness over the different long-term populations (Lenski & Travisano, 1994) could partly be explained by a recent study of Remold and Lenski (2004). They tested different mutations for the occurrence of epistasis by comparing their fitness effects over different genetic backgrounds. Half of the mutations showed strong epistatic interactions with their genetic background, in some cases even when these backgrounds had diverged in the same selective environment. This means that the fitness effect of a new mutation could be dependent on random historical events. This implies that differences that accumulated among the long-term populations may partly be the consequence of epistatic effects of mutations that were substituted earlier in the experiment. Complex genetic interactions, like pleiotropy (one mutation effects multiple traits) and epistasis (multiple mutations interact to determine the same trait) are a hallmark of rugged adaptive landscapes.

Wright's (1932) concept of the adaptive landscape is one of the most used metaphors in evolutionary biology. The landscape is a two or three dimensional depiction, in which the fitness is plotted against genotypes or phenotypes in a given environment. Natural selection will drive the population to the most nearby fitness peak. Therefore, the shape of the landscape will play a major role in the evolutionary process. The topology of the adaptive landscape is often described by 'smooth' *vs.* 'rugged', which is respectively, a single fitness peak *vs.* multiple local fitness peaks. In reality the adaptive landscape will be a highly dimensional interconnected space that is dependent on, not only the environment, but also on the genetic composition of the population itself. However, with using the metaphor of a mountain landscape, instead of a turbulent ocean, most studies indicate that this landscape is rugged (Colegrave & Buckling, 2005; Colegrave & Collins, 2008). The various long-term populations seem to have approached different fitness peaks of unequal height which generated between-population variation in fitness (figure 10c and 10d; Lenski & Travisano, 1994). In support, like explained above, large amounts of epistasis between mutations were observed (Elena & Lenski, 1997; Remold & Lenski, 2004). A rugged adaptive landscape implies a bigger role of chance events, such as the order in which mutations occur, because the fitness effects of these mutations are not additive. Another consequence of the rugged adaptive landscape, is that a population could get stuck at a suboptimal local fitness peak. So the topology of the adaptive landscape can constrain evolutionary adaptation (Colegrave & Buckling, 2005). Hence, the a population's evolvability could be improved by decreasing the ruggedness of the adaptive landscape (Colegrave & Collins, 2008). Finally, two additional points have to be made. Firstly, it is hard to interpret the ruggedness of a adaptive landscape because the connectedness is not only caused by point-mutations, but also by chromosomal rearrangements. Second, the presence of ruggedness does not contradict the parallel phenotypic changes observed in the previous paragraph. The phenotypic and even genomic parallelism shown in the previous paragraph implied a

strong adaptive evolution. The adaptive landscape has two layers, one from genotype to phenotype and the second from phenotype to fitness, therefore the eventual fitness improvement of a population is the sum of all their genetic and consequently phenotypic changes. The high similarity in phenotype was therefore not always the consequence of parallel genomic changes. For example, the *spoT* mutation found in Ara^{-1} population could not be found in Ara^{+1} population, although parallel changes were observed in global gene expression (Cooper *et al*., 2003). In addition, genomic parallelism was rarely found on the mutational level, for example the loss of maltose catabolism resulted from different mutational events in the same *malT* gene (Pelosi *et al*., 2006; Woods *et al*., 2006). Thus despite the many parallel changes at the phenotypic level, the differences at the genotypic level will locate the replicate populations at different places in the adaptive landscape metaphor. In addition, phenotypic differences underlying similar fitness advantages will relocate the different populations even further.

Genomic evolution

Papadopoulos and colleagues (1999) performed a fascinating study on genomic evolution in the long-term experiment. They analyzed the genomes of two long-term populations ([Ara^{-1}] and [Ara^{+1}]) after 10,000 generations of experimental evolution for restriction fragment length polymorphisms (RFLP), using seven different insertion sequences (IS) as probe. Most polymorphisms that could be detected with this approach, were the result of chromosomal rearrangements. A tremendous diversity accumulated within these populations, such that almost every individual had a different RFLP fingerprint at generation 10,000. So, even in this short time period the genome seem to be highly dynamic (figure 12). Considering the evolutionary dynamics two interesting results were found. First, the within-population genetic variation reached its highest level only after the rate of fitness increase had decelerated significantly. After this period, strong declines in genetic variation were observed periodically (figure 11b). These declines were probably caused by beneficial mutations that sweep to fixation and in the meantime eliminated all the genetic diversity, because each entire genome is one linkage unit (also called periodic selection; Atwood *et al*., 1951). Similar selective sweeps were also shown to result in step-like dynamics of fitness increase, as discussed in the section 'clonal interference'. The second interesting result was the discrepancy between genomic and phenotypic evolution (figure 11a and 6). In genomic evolution a linearly increasing genetic difference from the common ancestor was observed. In contrast to phenotypic evolution, the rate of this increase was not decelerating over time. This discrepancy could perhaps be explained by an increasing amount of hitchhiking mutations in later selective sweeps, because these sweeps need more time to become fixed due to their smaller selective advantages. In this way, the phenotypic fitness curve would reflect the amount of beneficial mutations that are fixed, while the genomic curve would reflect the total amount of fixed mutations. In addition, both populations that underwent similar fitness changes differed 3-fold in their rates of genomic evolution (a similar difference could be found in the within-population genetic variation).

Interesting parallels could be found in the evolutionary dynamics that is observed in these experimental studies with those observed in theoretical studies, we aim to illustrate this by giving one example (Wilke & Adami, 2002; Huynen *et al*., 1996). Huynen and colleagues (1996) studied the evolutionary dynamics in an artificial evolution experiment of RNA molecules. From a given RNA sequence the RNA secondary structure could be computed based on thermodynamic data. Like the robustness and neutrality experienced by an organism in the genotype to phenotype mapping and phenotype to fitness mapping, many sequences could underlie a similar structure. From this sequence to structure mapping, they calculated the fitness, which was the 'distance' to the target structure ($tRNA^{Phe}$). In other words, the closer a structure was to the most optimal form the fitter it was. So, in contrast to the above described experiments, the most adaptive form was known. The RNA population size was adjusted over time to keep the amount of RNAs fluctuating around a constant capacity, just like in the serial transfer regime (although not as strong as 100-fold). The robustness property of RNA folding was expressed by the term *neutral network*. A *neutral network* is a connection of RNA sequences that differ from each other by at most two mutations, but have an identical structure. So, in the experiments described above, a *neutral network* would be a connection of genomes that differ by at most a couple of mutations but convey a similar fitness advantage (including both layers of neutrality as mentioned above). So, not all the mutations that exist in a *neutral network* are neutral, but their connections through epistatic interactions are. In figure 13a the resulting evolutionary dynamics are shown. Like in experimental evolution (figure 13a), a step-like fitness increase could be observed (equal to the decrease in structure distance). Above the fitness curve a one dimensional projection of the sequence variation is shown (figure 13a). And similar to the dynamics observed when beneficial mutations sweep through a population (selective sweeps), every step-like increase in fitness resulted in a reduction of the genetic variation (figure 4c and 11b). In terms of the *neutral network* metaphor, the population drifts in sequence space over its *neutral network*, in which mutations occur that convey a fitness advantage that is entirely or almost neutral. In this way the sequence variation increases, until one variant in the population

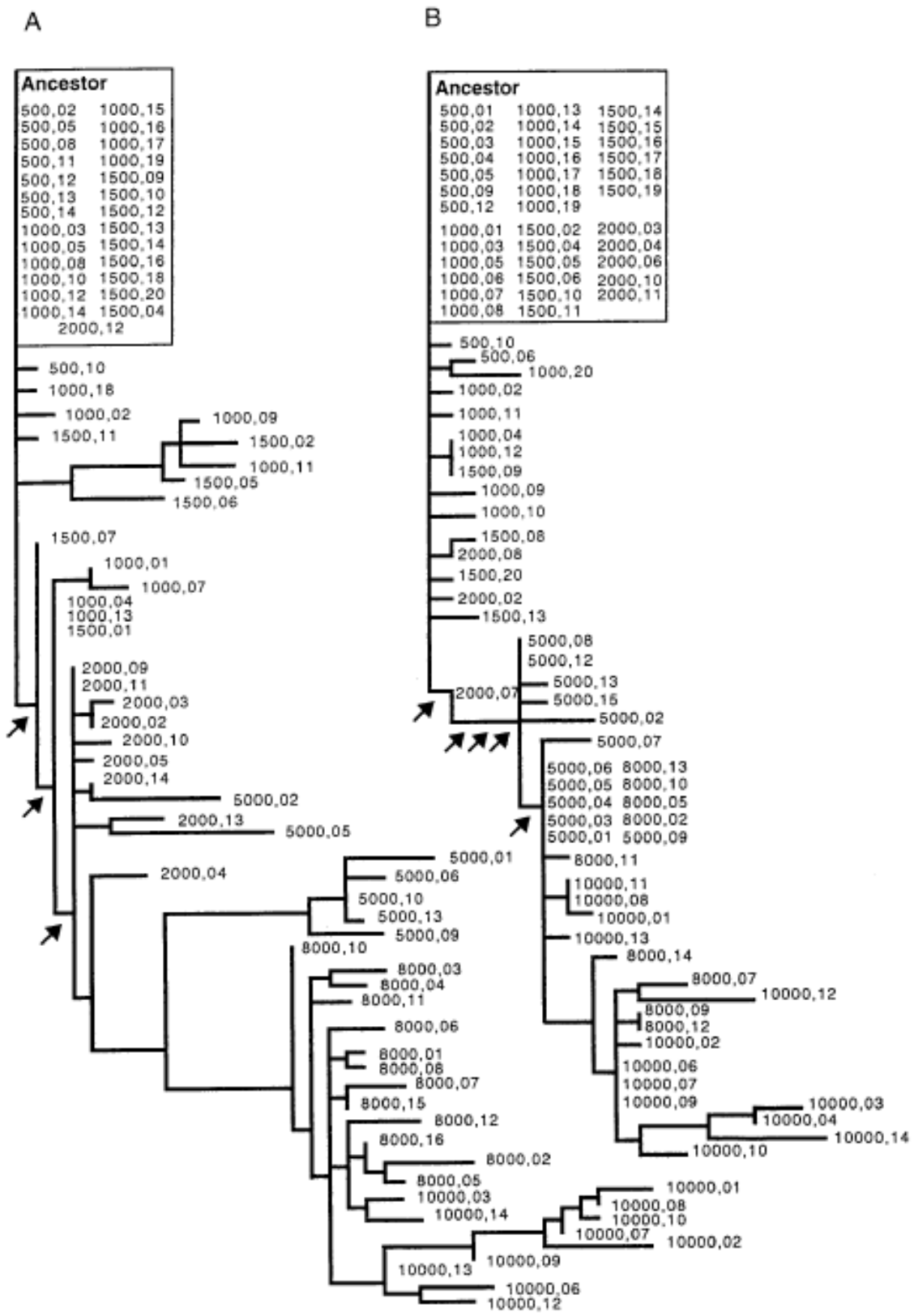


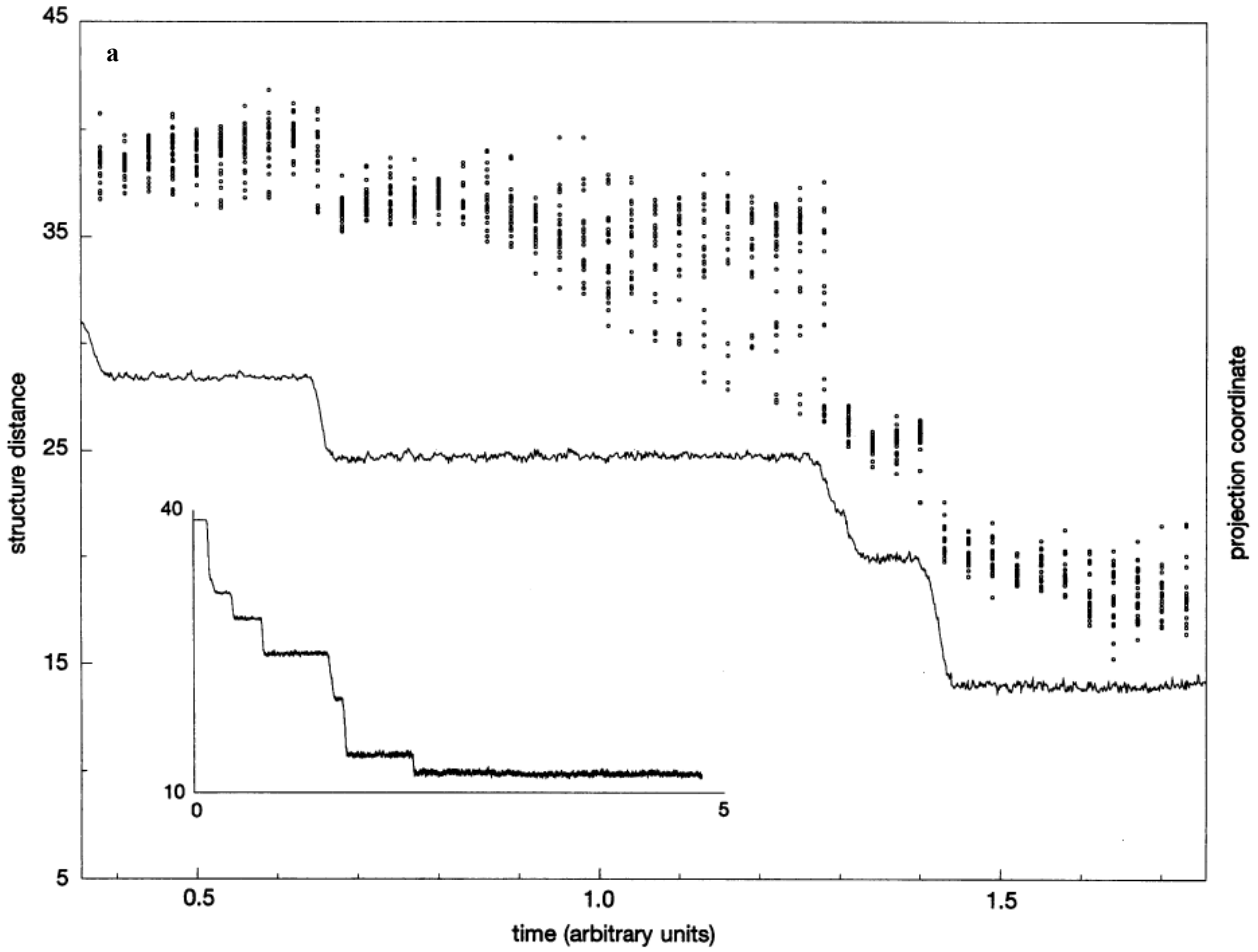
Figure 12. Phylogenies for clones from two evolving populations of *E. coli*, rooted by using the actual ancestral genotype. Phylogenies were inferred by parsimony from RFLP data obtained using IS elements as probes. Notation indicates the generation at which each clone was sampled, followed by an arbitrary number to distinguish clones from the same sample. Clones in the box with the ancestor were identical to the ancestor on the basis of there IS fingerprints. Arrows mark some of the pivotal mutations that were shared by all clones in every later sample. **a**, Ara^{+1} population; **b**, Ara^{-1} population. From Papadopoulos *et al*. (1999).

mutates to a more favourable *neutral network* and sweeps through the population until fixation. If one looks to the population structure at one point in time, multiple subpopulations could be observed (figure 13b). These subpopulations have mutations that convey similar fitness advantages (RNA structure) but independently undergo diffusion in sequence space over their *neutral network*. Some of these subpopulations are small and thereby prone to extinction in competition to those that are larger. So similar to clonal interference, the different subpopulation harbour different beneficial mutations which due to their full linkage with their genomic background will compete with each other. Eventually one population, and most probably the one with the highest fitness (the population that finds an entry point to a more favourable *neutral network*), will win this competition and the whole process starts all over again. Like in experimental evolution there was a discrepancy between phenotypic and genomic evolution (not shown), in which the rate of fitness increase decelerated more quickly than the rate of genomic evolution. Overall, although this model describes RNA evolution many similarities could be found with experimental evolution. These similarities arise from the *nonlinear* sequence to structure mapping and asexual propagation of the population. The *nonlinear* mapping between RNA sequence and structure, like the epistatic and pleiotropic interactions between genes, results in a rugged adaptive landscape which was also observed in experimental evolution. The asexual propagation results in clonal interference between the described subpopulations (figure 13b). The term *neutral network* may be deceiving, because it does not state that the genomic compositions are neutral, but refers to a connection of all genotypes that result in a similar fitness or phenotype. Therefore, the *neutral network* metaphor emphasizes the connectedness of the local fitness peaks in an adaptive landscape, by the presence of interwoven *neutral networks* in a high dimensional sequence space. It also emphasizes that a population should be regarded as a collection of subpopulations instead of a uniform mass of cells (at least during evolutionary adaptation).

Although the metaphorical views of adaptive landscapes and neutral networks could increase our insight in evolutionary dynamics, empirically they remain often restrictive and allow no further discrimination as 'rugged' *vs*. 'smooth' or the hazy image of interwoven neutral networks in a highly dimensional sequence space. Eventually, the ultimate goal would be to understand the dynamics by understanding all the occurring mutations and their phenotypic impacts on the complex interactions that make up the phenotype. A few of these mutations have been described in the previous paragraph, but an integrative view is still missing. So although experimental evolution happens in a quite simple environment the underlying processes can be complex. In the next section we will look to a nice case study of evolutionary dynamics, which is the occurrence of mutators.

Case study: mutators

Mutator-genotypes have an increased mutation rate throughout their genomes owing to mutations that disrupt some aspect of DNA replication or repair (Friedberg *et al*., 1995). Over the last two decades, many experiments have been performed to increase our understanding of how these mutators reach high frequencies in populations given their increased *genetic load* (Elena & Lenski, 2003). Some of these experiments studied spontaneously fixed mutators in experimental populations (Shaver *et al*., 2002; Sniegowski *et al*., 1997), while others deliberately introduced mutators and documented their effects on evolutionary dynamics (Chao & Cox, 1983; de Visser *et al*., 1999). Originally it was thought that mutators could accelerate adaptive evolution, however after closer examination this view seem to be implausible. First, there are relatively more deleterious mutations than beneficial mutations and therefore increasing the mutation rate also increases the *genetic load* (why otherwise would organisms have well functioning mismatch repair systems). Second, like shown in the section 'Clonal interference', increasing the mutational supply (product of population size and mutation rate) could only increase the rate of adaptive evolution when the supply is relatively small (de Visser *et al*., 1999). In the large populations where the spontaneous emergence of mutators is observed (Sniegowski *et al*., 1997) this mutational supply is not small (see introduction), and therefore it seem to be unlikely that the mutators increased the rate of adaptive evolution (this was also shown; Shaver *et al*., 2002). Third, to increase the supply of beneficial mutations the mutator genotype itself has to be common. So increasing the rate of adaptive evolution does not explain how the mutator genotype has *became* common. This last point was nicely shown by Chao and Cox (1983). They made different mixtures of normal and mutator phenotypes and watched for which relative frequency the mutator phenotype substituted the other. If the fraction of mutator phenotypes was substantially below that of normal phenotypes they always disappeared, but if it was above some critical frequency (mutator : normal = $7 \cdot 10^{-5}$) the mutators always took over. This shows that although each mutator cell had a higher *per capita* probability of acquiring the first beneficial mutation than a normal cell, the chance that subpopulation would acquire the first beneficial mutation would also depend on the population size. So, the chance that the subpopulation of a given phenotype would acquire the first beneficial mutation is dependent on its product of both subpopulation size and mutation rate, compared to that of the other subpopulation. When this product is above some critical threshold value the mutator population could take over. The observation that



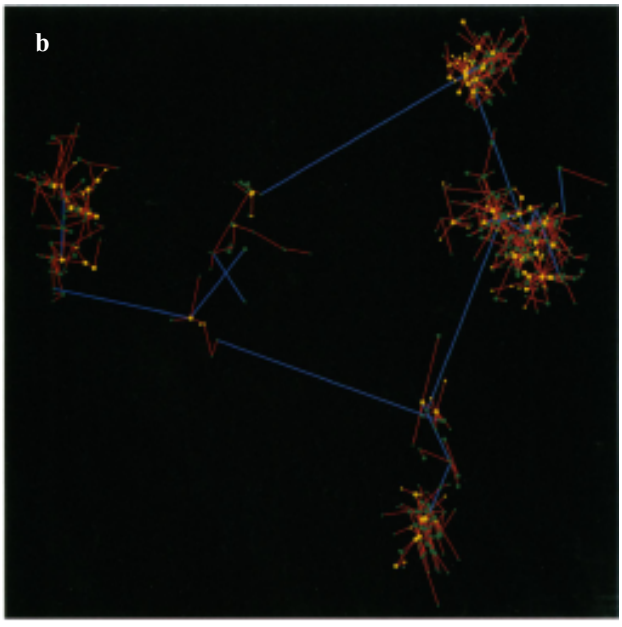


Figure 13. Dynamics in artificial evolution of RNA structures towards the optimal target structure $tRNA^{Phe}$, see for more details Huynen *et al*. (1996). **a**, Fitness dynamics of artificial evolution, which is equal to the distance between the current structure and the target structure. So the lower the distance the higher the fitness, therefore these artificial selection shows similar fitness dynamics as in the long-term experiment. Initiated with random sequences. Solid line, the populations average distance to the target structure plotted against time for a specific interval of the entire run (*Inset*). Superimposed series of dots render the evolution of the population structure over time. Dots at one time unit are a one-dimensional projection (1^{st} principle component) of the population of sequences present in >10 copies at that time, so the amount of genetic variation. **b**, population structure after 135 time units depicted in sequence space. The multidimensional sequence space is projected in two dimensions (1^{st} and 2^{nd} principle components of genetic variation) by using some theorems from distance geometry. Points represent 'individuals' in this sequence space containing a similar sequence. *Yellow* dots, populations fold into $tRNA^{Phe}$ target structure; *Green* dots, do not fold in target structures. Size of dot represents number of 'individuals' containing a particular sequence. Dots are connected by minimum spanning tree (short connections in *red*, long connections in *blue*). In total four big subpopulations (sub-quasi-species) could be discriminated. From Huynen *et al*. (1996).

mutator genotypes only took over when being relatively common, suggests that these mutators hitchhiked with some beneficial mutations that they produced, but were themselves not beneficial. But still, we do not know how mutator genotypes emerged in experimental populations. For example, in three of the long-term populations mutator genotypes emerged spontaneously within 10,000 generations ([Ara^{+3}], [Ara^{-4}], [Ara^{-2}]; Sniegowski *et al*., 1997). These mutators were the result of mutations in the methyl-directed mismatch repair pathway which resulted in a, between one and two orders of magnitude, higher

mutation rate. To understand how these mutator genotypes became so frequent one have to realize that the critical frequency of mutators, explained above, represents a stochastic and not a deterministic threshold (Elena & Lenksi, 2003). In other words, there is a certain chance that a mutator genotype will produce the next beneficial mutation. In addition, although mutators are outcompeted by normal cells owing to their *genetic load*, there will always be a quasi-equilibrium of mutators due to the constant mutational production of genotypes with mutations in their mismatch repair. Although this equilibrium is below the critical frequency, each beneficial mutation that is produced by one of the mutators is an opportunity for that mutator to hitchhike with it and increase in frequency. So the expected 10 to 20 beneficial substitutions that have occurred in every populations (Lenski, 2004), provided 10 to 20 chances for each population to be converted to a mutator. After a mutator is substituted the process is very unlikely to be reversed, until the population becomes so well adapted that the best mutation is one that reduces the *genetic load*. Thus overall, this nicely shows that evolution and its dynamics are the result of mathematical chance events that occur, of course these chance events are not arbitrary (the fitter the more likely an individual is to survive). Moreover, evolution does not necessarily optimize all cellular functions. For example, an increased mutation rate will often be deleterious due to its *genetic load*.

The long-term populations that became mutators showed no significant fitness gains in comparison to those that kept normal mutation rates (Shaver *et al*., 2002). Therefore this finding supports the study of de Visser and colleagues (1999), in which it was shown that the rate of adaptive evolution not always increases with an increasing mutational supply. Like explained above, small populations could benefit from higher mutation rates by shortening their 'waiting time' for a beneficial mutation to emerge. However, if populations become to small, higher mutation rates will be deleterious because selection loses his fight, in removing deleterious mutations, from genetic drift. In contrast, large populations will experience a 'speed limit' in their rate of adaptive evolution (which is independent of mutation rate) due to clonal interference. In summary, these experimental results have shown that in the rapid adaptive evolution that was experienced by the long-term populations, many beneficial mutations were substituted, and every substitution provided another opportunity for a mutator phenotype to emerge. Eventually, three mutator-populations emerged within the first 10,000 generations. However, the higher mutation rates that were experienced by these populations did not influence the rate of adaptive evolution (Shaver *et al*., 2002), which was probably the consequence of clonal interference.

A last interesting result to mention, is that high mutation rates can theoretical lead to 'survival of the flattest' (Wilke *et al*., 2001). Like explained in the previous section, a population can be seen as a cloud of genotypes travelling over a *neutral network* in sequence space. If mutation rate increases, selection would prefer such a population to go to the most densely packed region of the *neutral network*, because in this region most mutations are neutral and therefore the *genetic load* will decrease. The idea that selection prefers the most optimal solution for a cloud of genotypes that are interconnected by mutations, instead of an individual organism, is called the *quasi-species theory* (Eigen *et al*., 1988). In accordance with this theory, you would not expect the population to go to the highest fitness peak in the adaptive landscape ('survival of the fittest'), but to the flattest ('survival of the flattest'), even if this results in a lower replication rate *per* individual. This is because *quasi-species* that are located in flatter regions of the adaptive landscape, or more densely packed regions in the neutral network, will be more robust with respect to mutations. This hypothesis is only theoretically confirmed by using digital organisms (Wilke *et al.*, 2001) and like stated above, you would only expect to see this in experimental populations. If the best mutation left is a reduction of the *genetic load* (which of course, can also lead to a lower mutation rate). In conclusion, this last example shows that both metaphorical views discussed in the previous section, in combination with stimulations, can result in a new hypothesis which readily can be tested by experimental evolution (although it is not done yet). In addition, it shows that considering an individual as *the* selective unit in evolution, is maybe to restrictive when studying the dynamics of evolutionary adaptation.

CONCLUSION

In the last decades experimental evolution is used to increase our understanding of many evolutionary processes, like adaptive radiation, evolutionary adaptation and their dynamics. Other research approaches were only able to study these dynamics indirectly, by comparative analysis or theoretically modelling. In contrast, experimental evolution allowed researches to study evolutionary adaptation by natural selection directly (Lenski & Wiser, 2009). With using microorganisms for experimental evolution, the abilities became even larger. With microorganisms, derived genotypes were allowed to compete with ancestor genotypes to measure their relative fitness, microorganisms could be propagated for many thousands of generations and, in addition, microorganisms could genetically be manipulated to create isogenic lines that could be tested for particular mutations. These benefits evidently leaded to an increased understanding of the evolutionary process (Elena & Lenski, 2003; Lenski & Wiser, 2009).

Despite the simplest possible genetic and environmental conditions that are used in experimental evolution, complexity was generated from uniformity (Rainey *et al.*, 2000; Travisano & Rainey, 2000). These simplicities include environmental constancy, the absence of any other species, no sexual recombination and almost no genetic variation in the founding strain (Lenski, 2004). In general, adaptive radiation required two conditions: ecological opportunity and the proper selection regime. Ecological opportunity, or free niche space, was tested under two different environmental conditions, namely spatial and temporal heterogeneity. Especially the lack of spatial heterogeneity was shown to result in a loss of diversity (Kerr *et al.*, 2002; Rainey & Travisano, 1998). The locality of competition advantages was enough to generate overall frequency-dependent selection, which is one of the proper selection regimes for the maintenance of diversity (figure 1). Under the temporal heterogeneity of the long-term experiment, *cross-feeding* was shown to generate frequency-dependent selection (Turner *et al*., 1996). In one long-term population the frequency-dependent selection was significantly stronger than in the others, which resulted in a stable coexistence of two cell morphs, L and S (Elena & Lenski, 1997; Rozen & Lenski, 2000). This stable coexistence was the first polymorphism that was monitored for thousands of generations and showed highly complex temporal fluctuations (figure 4a). Later it was shown that both morphs formed monophyletic clades that independently adapted to their environment. The fluctuations could therefore be described as eco-evolutionary dynamics, in which the ecological interaction was altered over time due to the independent evolutionary adaptations of both clades (figure 4c and 5; Rozen *et al*., 2005). For future research it would be interesting to known which exact mutations were substituted in both clades and how these substitutions altered the ecological interaction. A better understanding of the impact of adaptive evolution on ecological relationships is important to unravel the ecological complexity and dynamics we see in nature. The discovery that such long-term complexities can evolve in simple model systems suggests that they might help us to illuminate macro-evolutionary events like speciation and extinction.

In the second paragraph we discussed evolutionary adaptation in the long-term experiment. It was shown that all population quite rapidly increased their fitness, relative to the ancestor, after being introduced in their new experimental environment. The evolutionary adaptation of glucose utilization automatically resulted in *ecological specialization*, in which fitness decreases when growing on alternative sugars (Cooper & Lenski, 2000). However, it should be noticed that using growth potentials on alternative substrates as the definition of niche breadth is still rather arbitrary. For example, you could also use the temperature range in which growth is possible (Cooper, 2002). The strongest evidence for evolutionary adaptation by natural selection was found in the many parallel molecular changes that were observed across the different long-term populations (Philippe *et al*., 2007). With a *top-down* approach by comparing different expression profiles, many parallel changes in expression could be found. These parallel changes resulted in the discovery of many parallel genetic modifications affecting two different levels of gene regulation: global regulatory networks and local regulons (Philippe *et al*., 2007). Most changes affecting the lower level could be explained quite straightforwardly, like the loss of ribose and maltose catabolic functions which were evidently costly to maintain (Cooper *et al*., 2001; Pelosi *et al*., 2006). However the consequences of mutations affecting the global regulatory networks were more difficult to explain thanks to the widespread pleiotropic effects that many of these functions can have. Both changes in DNA topology and the *stringent response* are involved in the transitions from lower to higher nutrient availability and *vice versa*. However, the importance of the discovered substitutions in these transitions could not be fully illuminated (Cooper *et al.,* 2003; Pelosi *et al*., 2006; Philippe *et al*., 2007). But, in general, it could be stated that the dynamics of these highly complex interconnected networks is important for bacterial adaptation across both physiological *and* evolutionary timescales. And although these networks are broadly studied from a molecular perspective, the evolutionary dynamics of these regulatory networks are largely unexplored. Experimental evolution can therefore make a first step in illuminating the evolutionary role of these networks, including their global regulatory genes. So, in total five different genes or operons have shown to be targets of selection in the long-term experiment: *rbs* (Cooper *et al*., 2001), *malT* (Pelosi *et al*., 2006), *topA* and *fis* (Crozat *et al*., 2005) and *spoT* (Cooper *et al*., 2003). Which together, assuming that their fitness gains are additive, could make up for a ~30% fitness increase relative to the ancestor.

The dynamics of evolutionary adaptation showed a dominant role for natural selection (Travisano *et al*., 1995), however this could not prevent the accumulation of variation due to chance events (figure 10c and 10d; Lenski & Travisano, 1994). In addition, many of these dynamics could largely be explained by clonal interference. The competition between genotypes containing different beneficial mutations imposes a 'speed limit' on the rate of adaptive evolution (de Visser, 1999). Sexual recombination would overcome this competition by combining the different beneficial mutations in one genome (Fisher, 1930; Muller, 1932). Colegrave (2002) tested this hypothesis by using the facultative sexual *Chlamydomonas reinhardtii* in an evolution experiment. He showed that the sexual populations on average had an increased rate of adaptation, and that this fitness benefit, relative to the asexual population,

was the biggest at large population sizes. Thus, in large populations sex seems to be able to release the 'speed limit' on adaptation set by clonal interference. One of the most remarkable results when looking to the evolutionary dynamics, is the enormous degree of dynamism of the genome (Schneider & Lenski, 2004), Papadopoulos and colleagues (1999) discovered this high degree of dynamism by using IS elements as markers for making RFLP analysis (figure 12). In addition, IS elements were successfully used for finding beneficial mutations and often were themselves beneficial, like the deletions observed in the *rbs* operon (figure 8b; Cooper *et al.*, 2001; Schneider & Lenski, 2004). However the impact of this dynamism on adaptive evolution is not fully understood, and therefore additional research herein is needed (Schneider & Lenski, 2004). When considering between-population variation of the independently evolved long-term populations, the adaptive landscape has to be rugged. This idea is supported by the discovery of epistatic interactions between different mutations (Elena & Lenski, 1997; Remold & Lenski, 2004). Although, besides the adaptive landscape, other metaphorical views can also be used to explain the observed dynamics, like *neutral networks*. In general, these metaphorical views are hard to comprehend with empirical data if you want to discriminate results further than 'smooth' *vs.* 'rugged'. However, they imply some important aspects in the physiological organisation of the cell for evolutionary adaptation. First, the nonlinear mapping from genotype to phenotype and from phenotype to fitness results in epistatic and pleiotropic interactions between different mutations. As a consequence the effect of a mutation is dependent on its genetic background. This is especially of importance for asexual microorganisms because their mutations are fully linked with their genome. Second, the mapping is robust, which implies that many different genomic compositions can underlie a similar phenotype or fitness. This neutrality can have important consequences for evolutionary adaptation, like is pointed out by the *neutral networks* (Huynen *et al.*, 1996). So in general, a better understanding of the genotype to phenotype mapping is needed to comprehend its impact on evolutionary adaptation.

Like stated in the introduction, we claim that experimental evolution studies gave one of the most robust evidences for Darwinian evolution, and we explained the three most important premises for adaptive evolution by natural selection. These premises were the presence of phenotypic variation, the non-randomly contribution of this variation to fitness and the (partly) genetic heritability of this phenotypic variation. All the observed beneficial mutations that were substituted satisfied these requirements, but the loss of ribose catabolism most clearly demonstrates the process of adaptive evolution. The ribose catabolic function was lost in all twelve independently evolving populations. However, before

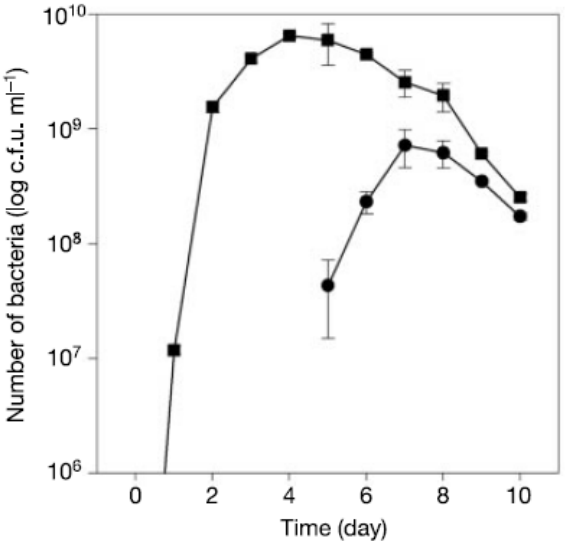


Figure 14. Emergence of defecting genotypes from WS during the course of selection in spatially heterogeneous microcosms. The defecting genotypes arise *de novo* by mutation and have a smooth colony morphology that enables them to be readily distinguished from the undulate WS genotypes. Values are means ± standard error of the mean of three replicates. Squares represent WS; circles represent defecting genotypes. c.f.u., colony-forming units. From Rainey & Rainey (2003).

the mutation, that resulted in the loss of ribose catabolism, was substituted in all populations a polymorphism existed between the ancestor phenotype and the derived phenotype, with respectively, a functional ribose catabolism (Rbs^+) and a loss of ribose catabolism (Rbs^-) (1st requirement; figure 7a; Cooper *et al.*, 2001). This variation contributed non-randomly to an individual's fitness. Individuals that lost their ribose catabolic function had a fitness gain of about 1% to 2% in comparison to individuals with a functional ribose catabolism (2nd requirement; figure 7b). And lastly, it was found that the phenotypic variation was heritable, because the loss of the ribose catabolic function was caused by an *IS150-element-induced* deletion in the *rbs* operon (3rd requirement; figure 8a and 8b). As can be expected when fulfilling all these requirements, the Rbs^- phenotype replaced the Rbs^+ phenotype in all the twelve populations and therefore evidently demonstrates adaptive evolution by natural selection.

In general, adaptive evolution in the long-term experiment resulted in the loss of catabolic functions (Cooper *et al.*, 2001; Pelosi *et al.*, 2006) and most of the parallel changes observed within the microarray analysis (Cooper *et al.*, 2003) and global protein profiling (Pelosi *et al.*, 2006) involved decreased expressions. In other words, adaptation did not result in constructive evolution in which new catabolic functions evolved (as can be expected in a glucose minimal medium). However, experimental evolution could give an important contribution to our understanding of constructive evolution (Fani & Fondi, 2009; Lenski & Wiser, 2009). Mortlock and

colleagues (1992) showed that under laboratory conditions bacterial catabolic evolution can lead to new catabolic activities due to the recruitment of pre-existing enzymes. Their results supported the *patchwork hypothesis* (Jensen, 1976) in the origin of metabolic pathways. This hypothesis can be explained in two steps. First, an ancestral enzyme with low substrate specificity is able to marginally catalyze different substrates. Second, duplication of the gene encoding this enzyme and subsequent divergence results in an second enzyme that has increased specialization for one of the substrates. In other words, new metabolic functions can evolve by 'borrowing' gene products that are normally used for other functions but have a marginal catalytic effect on the new substrate. In turn, this can favour gene duplication that stimulates the evolution of a higher substrate specificity (Lenski & Wiser, 2009). In addition to constructive evolution, experimental evolution could contribute to many more fundamental questions in evolutionary biology, like: why did sex evolved? why do organism cooperate? how did multicellularity evolved? etc. Many studies already started to tackle these difficult questions (Colegrave, 2002; Cooper *et al.,* 2005; Lenski & Wiser *et al.,* 2009; Rainey & Rainey, 2003; Travisano & Velicer, 2004; Velicer *et al*., 2000). For example, Rainey and Rainey (2003) tested the evolution of cooperation and conflict in *P. fluorescens*. Like discussed in the paragraph 'Polymorphism and diversification', Rainey and Travisano (1998) observed rapid adaptive radiation of *P. fluorescens* into three morphs: smooth colony morph (SM), wrinkly spreader (WS) and fuzzy spreader morph (FS). Diversity was maintained thanks to frequency-dependent selection and spatial heterogeneity. Rainey and Rainey (2003) studied the cooperation of the wrinkly spreader morph (WS), which inhabits the air-liquid interface. WS cells over-produce an adhesive polymer which is costly for the cell, but nevertheless the trait spreads by kin selection because it promotes colonization of the air-liquid interface, which is beneficial for the group. As could be expected, after a few days defector genotypes evolved that cheated by living at the air-liquid interface without over-producing adhesive polymer (figure 14). The relative increase of defector genotypes causes the WS mat to collapse. However, the WS cells are a persistent feature because after every collapse they emerge afresh and are maintained by frequency-dependent selection. Overall, this example shows everything you would expect to see in the evolution of cooperation (Maynard Smith & Sazthmáry, 1995). The main point of this example, is the ease in which theoretical predictions can be tested with experimental evolution and, in addition, it shows that experimental evolution can be used to study complex problems.

The combinational approach of using theoretical predictions, or simulations, and compare these with experimental results have shown to be useful (Gerrish & Lenski, 1998; Kerr *et al.*, 2002; Rozen *et al*., 2002; de Visser & Lenski, 2002). In the last years the experimental evolution approach is extended by using digital organisms (Adami, 1998; Adami, 2006; Adami & Wilke, 2004; Chow *et al*., 2004; Lenski *et al.*, 1999; Wilke & Adami, 2002). Digital organisms are self-replicating computer programs that live in a controlled computer environment. These digital organisms can be used to create new hypothesis, but they can also be used to confirm existing hypothesis like seen with the 'survival of the flattest' hypothesis (Wilke *et al*., 2001). Another example is that of Lenski and colleagues (2003), who studied the evolutionary origin of complex features by using digital organism. And similar to *patchwork hypothesis* (Jensen, 1976) they found that complex features evolved by modifying existing structures and functions. The advantages of using digital organisms to study evolutionary adaptation are the extreme short generation times and the unprecedented accuracy in which measurements could be taken. These advantages are quite similar to the advantages of experimental evolution in comparison with other empirical approaches. However, testing evolutionary problems with digital organisms could only complement, but never replace, experimental evolution. Namely, research with digital organisms is restricted to abstract questions about fundamental principles. But we cannot gain inside in biochemical pathways using digital organisms, for example, these organisms do not have transcription and translation. Moreover, the design choices that enter the construction of the digital world in which evolution happens could influence its outcome, and therefore you would always require empirical support to be conclusive.

In conclusion, experimental evolution, although studying evolution in a simplified environment, can increase our understanding of the biocomplexity as we see in nature. Over the last decades, experimental evolution has developed to one of the key studying approaches in evolutionary biology, and showed one of the most convincing evidences for adaptive evolution by natural selection (Elena & Lenski, 2003; Lenski & Wiser, 2009). However many challenges remain to be solved, even in this simplified environment the uttermost complexities, like the highly complex global regulatory networks, are not understood. It is evident that these complexities play a significant role in evolutionary adaptation. In addition, many micro- and macro-evolutionary events like complex ecological communities, speciation, extinction, the evolution of sex, the evolution of multicellularity, the evolution of cooperation, etc. have to be challenged before we can fully understand what we see in nature. Experimental evolution, in combination with mathematical simulations and phylogenetic data, yet keep our expectations high in facing these problems which are considered as the most fundamental in evolutionary biology.

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GLOSSARY OF TERMS

Bisexual
Bisexual species are species that contain two genders. Mostly called the male and the female.

Bottleneck
A severe reduction in population size during a relative short period of time, which results in a loss of genetic variation. As a consequence the role of genetic drift increases and the role of natural selection decreases.

Clonal interference
The competition of multiple beneficial mutations that are fully linked to the genomic background in which they occur. The lack of genetic recombination in asexual organisms, makes it impossible to combine all beneficial mutations in one genome.

Demographic trade-off
A trade-off that results in a population structure that changes over time, in which one part of the population flourishes in 'feast' period and the other in the 'famine' period. For example a metabolic trade-off between maximum growth rate and substrates affinity, which makes it impossible to be successful during both 'feast' and 'famine'.

Ecological specialization
A metabolic trade-off whereby the increased adaptation of an organism to a specific ecological environment results in a reduction of fitness in other ecological environments. Both *antagonistic pleiotrophy* or *mutational accumulation* (terms explained in the text) can be responsible for this trade-off.

Effective population size
The size of an ideal random mating population that would lose genetic variation via genetic drift at the same rate as is observed in the actual population (Freeman & Herron, 2007).

Epistasis
A non-additive interaction between multiple mutations on an individual's phenotype. Such that their combined effect on the phenotype deviates for the sum of their individual effects.

Genetic drift
The change in frequency of genotypes in a population that is caused by chance differences in survival and reproduction, and therefore results in non-adaptive evolution. Under the experimental conditions sampling errors will cause such changes in frequency.

Genetic load
A reduction in fitness thanks to the occurrence of deleterious mutations.

Latent selection potential
Genetic variation that cannot immediately be used by natural selection (*latent*), but when environmental conditions change, increases the rate of evolutionary adaptation. For example, the accumulation of neutral genetic variation of which the neutrality is dependent on the environmental conditions.

Mixis
The recombination of genetic information from multiple sources into a new genotype. For example, sexual reproduction and horizontal gene transfer.

Neutral network
A connection of multiple genomic sequences that differ by at most a couple of mutations but convey a similar fitness advantage *or* phenotype.

Niche exclusion principle
The statement that no two species can occupy the same niche. Similar to Gause's (1934) *competitive exclusion principle*, in which he stated that "the number of species cannot exceed the number of distinct resources".

Pleiotropy
A single gene that influences multiple phenotypic characteristics.