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BACTERIAL EVOLUTION: RANDOM OR SELECTIVE?

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INTRODUCTION

The publication of *The Origin of Species* by Charles Darwin in 1859 constitutes a fundamental milestone in the history of science. In this book, Darwin builds up his theory of evolution based on the objective statements that living organisms change, that changes are transmitted to the progeny and that reproduction of organisms frequently gives rise to progenies that are too numerous to permit the survival of all the individuals. Darwin then concludes that in general, those individuals that change in such a way that their fitness to the environment increases will have a better chance to survive and reproduce. Thus, variations that are beneficial will gradually accumulate by simple natural selection.

What struck most the world at large was not the realization that living organisms evolve; after all, a transformist theory had been advanced four decades earlier by the French naturalist Jean Baptiste Lamarck in his *Histoire naturelle des animaux sans vertèbres* but the substantial differences between the theories advanced by both scientists. According to Lamarck, during their lifetime organisms undergo changes that favor their adaptation to the environment. These changes, which are influenced by the environment, are then transmitted to the offspring. Lamarck also stated that the evolutionary paths of the different species are independent of each other and that evolution follows a natural path towards perfection. In contrast, Darwin proposed that there is no such tendency to perfection. Rather, variation of living organisms is gradual, passive, spontaneous, with no destination. Favorable traits would be transmitted through the progeny, whereas those that are detrimental would tend to disappear. Moreover, in sharp antagonism with Lamarck, Darwin proposed the theory of common descent.

That variation (or mutation, as we call it now) arises spontaneously with no influence from the environment and without regard for utility has been elegantly shown by Luria & Delbruck[1] and by Lederberg & Lederberg,[2] in studies that are considered classic contributions to the field of molecular genetics. What these authors described correspond to mutations that are said to be growth-dependent, because they exhibit a definable relationship to cell division and are considered to result from random errors of the DNA replication machinery.[3] Does this undeniable fact imply that there is no variation promoted by the environment, as Lamarck had put forward? For a long time, growth dependent mutations were considered to be the primary cause of Darwinian evolution and even today it is so portrayed in the non specialized literature. However, some decades ago, researchers began to observe mutations that arise in non-growing, nutritionally deprived bacterial cultures that were subjected to non lethal selective pressure. Unexpectedly, these mutations appeared to have arisen with certain specificity in order to allow a better adaptation to the stressful environment.

Studies at the molecular level later showed that the mechanisms implicated in adaptive genetic change offer a much higher versatility of variation than the sole growth-dependent mutations attributed to errors of the DNA replication machinery. Although any one would think that most mutations are expected to be detrimental, an increase in variation is needed to allow some members of the population to arrive at a phenotype suitable for survival and proliferation in the new environment.

A CHALLENGE TO RANDOM AND GRADUAL MUTABILITY

The first hint of mutations in non-growing cells was obtained by Ryan about fifty years ago.[4] He observed that cultures of *his*$^-$ *Escherichia coli* auxotrophs inoculated into medium lacking histidine continued to produce

[1] Luria, S., Delbruck, M. Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28, 491-511, 1943.

[2] Lederberg, J., Lederberg, E.M. Replica plating and indirect selection of bacterial mutants. *J Bact* 63, 399-406, 1952

[3] In this case, the term random is used in a loose way, since geneticists are well aware that an average genome possesses hot spots for spontaneous mutations.

[4] Ryan, F.J., Wainwright, L.K. Nuclear segregation and the growth of clones of spontaneous mutants of bacteria. *J. Gen. Microbiol*. 11, 364-379, 1954.

his$^+$ revertants during a period of ten days after inoculation. He did not investigate whether other mutations also occurred, although he confirmed that the revertants were not slowly growing mutants previously present in the inoculum. A couple of decades later, Hall & Clarke found that a deletion mutant in the *lacZ* gene encoding β-galactosidase, when incubated for several days in the presence of lactose, reverted to a phenotype that allowed metabolism of this sugar.[5] The *lacZ* gene encodes an enzyme called β-galactosidase, which breaks down lactose into its components glucose and galactose. The new phenotype was the result of two mutations in an operon called *ebg* (for evolved β-galactosidase), which specifies a second β-galactosidase of unknown function. A mutation in the gene *ebgA* activates the enzyme, whereas a second mutation in the gene *ebgR* inactivates the repressor of the operon. Considering that either of the single mutations did not represent any advantage to the cell, it is remarkable that the double mutants arose with a frequency much higher than expected. Later, in separate studies, Shapiro[6] and Cairns *et al.*[7] investigated reversion rates in *E. coli* cells with bacteriophage Mu inserted into a fusion between the regulatory segment of the arabinose operon and the *lacZ* gene.[8] In this system, excision of Mu prophage led to fusion of *araB* to *lacZ*, yielding a Lac$^+$ cell as long as arabinose was also present to act as an inducer. The evidence showed that incubation for several days in both sugars, but not in either of them alone, led to the appearance of colonies in which Mu had been excised, whereas cultures grown without starvation produced none.

Other examples followed. Benson incubated bacteria in medium containing maltodextrins as the only carbon source. Normally, these high molecular weight polymeric substances do not trespass the cell membrane. However, bacteria underwent mutations in the gene encoding an outer membrane porin that allowed their ready entry into the cell.[9] In

[5] Hall, B.G. and Clarke, N.D. Regulation of newly evolved enzymes. III. Evolution of the *ebg* repressor during selection for enhanced activity. *Genetics* 85, 193-201, 1977.

[6] Shapiro, J. Observations on the formation of clones containing araB-lacZ cistron fusions. *Mol Gen Genet* 194, 79-90, 1984.

[7] Cairns, J., Overbaugh, J., Miller, S. The origin of mutants. *Nature* 335, 142-145, 1988.

[8] Both the arabinose and lactose operons are missing in this strain, which therefore is ara- and lac-. However, upon deletion of the intervening Mu prophage, it can grow on lactose provided arabinose is present.

[9] Benson, S.A., Partridge, L., Miller, S. Is bacterial evolution random or selective? *Nature* 336, 21-22, 1988.

turn, Hall pursued his work analyzing other systems. One of them required double mutations for utilization of β-glycosides, namely, excision of an insertion sequence and a point mutation.[10] Incubation in solid medium, only when containing substrate, promoted both mutations allowing its metabolism. Hall also tested for the first time the production of mutations in anabolic genes.[11] Two *E. coli* strains, each one possessing single point missense mutations in genes encoding enzymes for the synthesis of tryptophan (the *trp* operon), exhibited elevated reversion frequencies during starvation of this amino acid. Reversions in the *trp* operon did not take place when cells were starved for cysteine and mutation rates in other loci did not increase during tryptophan starvation. Therefore, the increased reversion rate appeared to be specific to conditions where the mutations were advantageous. In a subsequent study,[12] the author showed that a strain carrying two missense mutations in the *trp* operon reverts 10^8 times more frequently than would be expected if the two mutations were the result of independent events.

At the same time of the latter studies by Hall, one of the most paradigmatic papers in the field was published by Cairns and Foster.[13] These authors measured the reversion of a frameshift rather than a point mutation in the Lac operon of *E. coli*, which in this case is carried in an F' conjugative plasmid. The strain, called FC40, is deleted for the Lac operon on its chromosome and at the same time is resistant to the RNA polymerase inhibitor rifampicin due to a mutation in the chromosomal *rpoB* gene. The mutants were found to vigorously revert to Lac^+ (about one revertant per 10^7 cells per day) when plated on lactose minimal medium, whereas no reversion to the wild type rifampicin resistance phenotype was observed. Conspicuously, Lac^+ mutants did not arise in the absence of selection, i.e., when lactose was not present in the medium.

One of the most striking features of these early studies was that the increased frequencies of the advantageous mutations were not accompa-

[10] Hall, B.G. Adaptive evolution that requires multiple spontaneous mutations. I. Mutations involving an insertion sequence. *Genetics* 120, 887-897, 1988.

[11] Hall, B.G. Spontaneous point mutations that occur more often when advantageous than when neutral. *Genetics* 126, 5-16, 1990.

[12] Hall, B.G. Adaptive evolution that requires multiple simultaneous mutations: mutations involving base substitutions. *Proc Natl Acad Sci USA* 88, 5882-5886, 1991.

[13] Cairns, J., Foster, P.L. Adaptive reversion of a frameshift mutation in *Escherichia coli*. *Genetics* 128, 695-701, 1991.

nied by mutations at other loci.[14] This apparent selectivity, in open contradiction with the prevalent doctrine of randomness, astounded researchers in the field. For example, Cairns *et al.*[7] dared to state that 'In this paper...we describe some experiments suggesting that cells may have mechanisms for choosing which mutations will occur'. Also: 'This experiment suggests that populations of bacteria in stationary phase have some way of producing (or selectively retaining) only the most appropriate mutations'. Cairns even proposed molecular processes that 'could, in effect, provide a mechanism for the inheritance of acquired characteristics'. One of them was completely ground-breaking, since it implied information transfer from protein to DNA. According to this model, a reverse transcriptase instructed by *some element* that monitors the protein products would retrotranscribe an mRNA variant encoding a useful protein. Cairns referred to these mutations as adaptive,[13] while they were called 'directed' mutations by the editors of *Nature*[15] and 'a unicorn in the garden' by Franklin W. Stahl.[16]

Undoubtedly, this idea challenged the traditional thinking about spontaneous mutation, although the possibility of non randomness in variation had never been completely abandoned. In fact, Delbruck himself had previously noted the distinction between selecting for phage resistance versus selecting for carbohydrate utilization, stating that 'in view of our ignorance of the causes and mechanisms of mutations, one should keep in mind the possible occurrence of specifically induced adaptive mutations'.[17] A. Weismann, the father of neo-Darwinism, stated late in his career that directed variation must be invoked to understand some phenomena, as random variation and selection alone are not sufficient explanation. In turn, the

[14] Typically, in these studies, a mutant bacterial strain that requires a nutrient is plated on solid medium that contains a very limiting supply of the nutrient. When the nutrient is exhausted, there is a sparse population of bacteria on the agar and further growth cannot occur unless a known mutation reverts. The first observable colonies are considered to be spontaneous mutants that were present in the population prior to plating. Further incubation of the plates for several days up to a month reveals the continuous appearance of new colonies in numbers that cannot be predicted by the Luria&Delbruck test. These late appearing colonies that arise in a non-growing population of bacteria that are subjected to a nutritional stress are said to result from adaptive mutation.

[15] Cited in Foster, P.L. Adaptive mutations: Has the unicorn landed? *Genetics* 148, 1453-1459, 1998.

[16] Stahl, F.W. A unicorn in the garden. *Nature* 335, 112-113, 1988.

[17] Delbruck, M. Heredity and variations in microorganisms. *Cold Spring Harbor Symp. Quant. Biol*. 11, 154 – , 1946.

eminent geneticist T. Dobzhansky expressed by mid 20th century that 'The most serious objection to the modern theory of evolution is that since mutations occur by chance and are undirected, it is difficult to see how mutation and selection can add up to the formation of such beautifully balanced organs as, for example, the human eye'.[18] Interestingly, in a speculative paper published earlier than Cairns' work, Fitch had stated that 'because mutations are advantageous during stressful times but genome wide mutagenesis would be deleterious, organisms probably have evolved a mechanism for selectively mutating only the genes of relevance'.[19]

As expected, the possibility that certain mutations in bacteria that were in stationary phase and subjected to non-lethal selective pressure might occur at higher rates when advantageous gave rise to a deep controversy.[20] This new type of mutation that came into sight more often when beneficial than when neutral appeared to vindicate the Lamarckian idea that the environment influences variation to improve adaptation. In this case, however, changes would obviously not occur as a result of use or disuse of a particular organ. Instead, they might perhaps arise from selection based on the presence of molecular variations within cells. On the other hand, one of the main arguments used by the supporters of adaptive mutations was that the classical experiment of Luria & Delbruck could not show the appearance of mutations during selection, since their protocol involved a lethal selection assay (resistance to bacteriophage T1). This assay gave no chance to detect additional mutations in cells that had not become resistant to viral infection.

Are adaptive mutations really directed?

The first hint that there was not reverse information flow that would instruct the cell how to mutate to attain successful survival was obtained by a reversion of an amber mutation in an episomal *lacZ* gene, both through

[18] Quotations by Weismann and Dobzhansky taken from: Wright, B.E. A biochemical mechanism for nonrandom mutations and evolution. *J Bact* 182, 2993-3001, 2000.

[19] Fitch, W.M. The challenges to Darwinism since the last centennial and the impact of molecular studies. *Evolution* 36, 1133-1143, 1982.

[20] See for example letters by several scientists and rebuttals in *Nature* 336, 21-22, 1988; *Nature* 336, 525-528, 1988 and *Science* 269, 285-289, 1995. Also: Lenski, R.E., Slatkin, M., Ayala, F.J. Mutation and selection in bacterial populations: alternatives to the hypothesis of directed mutation. *Proc. Natl. Acad. Sci. USA* 86, 2775-2778, 1989; Lenski, R.E., Mittler, J.E. The directed mutation controversy and neo-Darwinism. *Science* 259, 188-194, 1993.

intragenic mutations that eliminate the stop codon and by extragenic creation of a tRNA suppressor.[21] The latter necessarily had to be random, since there was no relationship between lactose metabolism and a chromosomal gene encoding a tRNA. In a subsequent study, Foster tested the mutability of a second gene (tet^S) also present in the plasmid harboring the *lacZ* gene mutant. She found that upon selection in lactose, tet^R mutants appeared at about the same rate as Lac^+ mutations.[22] These results showed clearly that selection was unnecessary for obtaining mutations in stationary phase, as originally thought. The concept of adaptive mutation was hence adjusted to mean those mutations that occur in non dividing cells during selection and are specific to the selective pressure. Mutants that arise in non dividing cells and that are either not adaptive, or have not yet been shown to be adaptive, were called stationary phase mutations.[23] Later, other Lac^+ revertants of the *E. coli* strain FC40 were found to carry mutations that were not related to selection.[24,25]

In turn, specificity of reversion of trp^- mutants was shown by the lack of reversion in cultures starved for other amino acids, as well as by the lack of appearance of other mutants during starvation for tryptophan. Out of 110 trp^+ revertants, Hall found only two carrying additional mutations.[26] However, he was somewhat cautious in the interpretation of these results: he stated that the explanation for the apparent influence of the environment in the selectivity of mutation did not necessarily have to be found in the two extreme choices that had been so far considered, namely randomness or directedness. He proposed to adopt the concept of 'Cairnsian' mutation to imply those sequence changes that occur with a higher probability when they are advantageous than when they are neutral. Later, citing a personal communication by J. Cairns, he speculated that the specificity could be

[21] Foster, P.L, Cairns, J. Mechanisms of directed mutation. *Genetics* 131, 783-789, 1992.

[22] Foster, P.L. Nonadaptive mutations occur on the F' episome during adaptive mutation conditions in *Escherichia coli*. *J Bact* 179, 1550-1554, 1997.

[23] Foster, P.L. Adaptive mutation: the uses of adversity. *Ann Rev Microbiol* 47, 467-504, 1993.

[24] Rosche, W.A., Foster, P.L. The role of transient hypermutators in adaptive mutation in *Escherichia coli*. *Proc Natl Acad Sci USA* 96, 6862-6867, 1999.

[25] Torkelson, J., Harris, R.S., Lombardo, M.J., Nagendran, J., Thulin, C., Rosenberg, S.M. Genome-wide hypermutation in a subpopulation of stationary cells underlies recombination-dependent adaptive mutation. *EMBO J* 16, 3303-3311, 1997.

[26] Hall, B.G. Spontaneous point mutations that occur more often when advantageous than when neutral. *Genetics* 126, 5-16, 1990.

explained by either selective capture or selective generation.[27] The former mechanism implies that mutations take place randomly and continuously during prolonged selection, but only those that are useful are captured by replication or recombination and immortalized by growth. Useless mutations have no way to express themselves. Selective generation, on the other hand, implies that sequence changes occur only in genes that are being actively transcribed. Indeed, one likely mechanism for directing mutations to specific genes requires their active transcription under nutritional deprivation (see below).

Systems involving mobile genetic elements represent a different situation. In the case of prophage Mu excision from the *araB-lacZ* fusion to allow growth on lactose when arabinose is also present,[6,7] the specificity of genetic variation is obvious. In the *egb* operon, it has been established that the gene *ebgR* encoding the repressor is a hot spot for the insertion of the mobile element IS30, whereas in the *bgl* operon the gene *bglF* reverts to wild type by excision of IS103. The latter event precedes mutations in the promoter (*bglR*), which will eventually allow growth in β-glycosides. In either of these situations, where movement of the mobile elements is stimulated by stress (see below), directedness could be explained by selective capture.

In spite of these clarifications, the controversy regarding the directedness of mutations followed for several years.[28] Even recently, Roth *et al.*[29] have been particularly critical in accepting that selection stimulates formation of new mutations. These authors prefer to think that what selection actually does is to allow faster growth of pre-existing mutants, with the parent strain remaining unable to grow due to the stringent conditions of the medium. However, the recent unraveling at the molecular level of several mechanisms involved in stress induced mutagenesis seems to leave no room for a controversy. It is now understood beyond doubt that stressful environments induce in bacteria genomic instability which results in mutants that are fitter than the parent strain to the adverse conditions.

[27] Hall, B.G. Adaptive mutagenesis: a process that generates almost exclusively beneficial mutations. *Genetica* 102/103, 109-125, 1998.

[28] See for example the series of papers by Rosemberg & Hastings, Ross & Andersson and Foster, with the corresponding rebuttals, in *J Bact* 186, 4838-4863, 2004.

[29] Roth, J.R., Kugelberg, E., Reams, A.B., Kofoid, E., Andersson, D.I. Origin of mutations under selection: The adaptive mutation controversy. *Annu Rev Microbiol* 60, 477-501, 2006.

A stressful environment induces adaptive mutations

Cells have different DNA repair pathways that are responsible for correcting sporadic mistakes arising as a result of DNA polymerase errors or through chemical modification of the bases. Therefore, mutations in the DNA are supposed to be transient, because they are normally corrected. However, under stressful conditions, these repair pathways are either down-regulated or become overwhelmed while taking care of abundant DNA damage.

There are several stress responses that intensify genetic variation in bacteria.[30,31] As mentioned previously, the molecular mechanisms leading to mutations in these pathways are different from those taking place in growing cells. All the previous findings of adaptation in non-growing cultures can now be interpreted under the light of one of these mutagenic pathways. In some cases, they may give rise to localized sequence changes, which have the advantage of avoiding non-adaptive mutations. The apparent selectivity observed in some of the laboratory studies may explain the original interpretation of directedness.

Perhaps the most thoroughly studied mutagenic pathway is the SOS response.[32] It is induced by extensive DNA damage, by cell saturation in rich medium, exposure to antibiotics and in aging colonies. About 30 genes encoding functions related to DNA metabolism are under the control of LexA repressor. Among them are those specifying DNA polymerases IV (*dinB*) and V (*umuC,D*), which are able to replicate damaged DNA although with low fidelity. Normally, the genes of the pathway are silent or are expressed at very low levels. The SOS response is triggered when the stressful environment induces RecA-dependent auto-proteolysis of LexA. If cells are proliferating, the two error prone polymerases increase the mutation rate by competing with the accurate DNA polymerase III, which replicates the chromosome under normal conditions. In non-growing cells, partial DNA synthesis by the mutagenic enzymes takes place during repair or recombination events. Some of the mutants arising will have a selective advantage for survival.

[30] Foster, P.L. Stress responses and genetic variation in bacteria. *Mutation Res* 569, 3-11, 2005.

[31] Foster, P.L. Stress-induced mutagenesis in bacteria. *Crit. Rev. Biochem. Mol. Biol.* 42, 373-397, 2007.

[32] Schlacher, K., Goodman, M.F. Lessons from 50 years of SOS DNA damage induced mutagenesis. *Nature Rev Mol Cell Bio* 8, 587-594, 2007.

Another important pathway is the general stress response.[33] In this case, the controller protein is not LexA but RpoS, a sigma factor (σ^S) that replaces the vegetative sigma factor σ^{70} of RNA polymerase. Sigma factors are critical for gene expression, since they are responsible for the selectivity of transcription by RNA polymerase. Nutrient limitation or stationary phase of growth results in the accumulation of polyphosphate (PolyP). This compound causes an elevation in the titers of σ^{70}, leading to higher levels of the error-prone DNA polymerase IV or to an inhibition of the expression of enzymes belonging to the mismatch repair (MMR) pathway. Both effects contribute to raise the adaptive mutation rate in bacteria.

Amino acid starvation also causes the buildup of (p)ppGpp, a phenomenon commonly known as the stringent response.[34] This rare nucleotide inhibits initiation of DNA replication and influences the selectivity of transcription by RNA polymerase. For example, it down regulates the synthesis of rRNAs and tRNAs while it also collaborates in raising the levels of RpoS. In addition, (p)ppGpp up regulates the operons for amino acid biosynthesis, which are normally subjected to end-product repression. It is well known that genes under transcription are more liable to mutate due to their partial single stranded character.[35] Thus, starvation for a specific amino acid makes its synthetic operon more susceptible to mutations. This may be the explanation for the 'directedness' observed by Hall in the reversion of the *trp* mutants.[11,12] DNA damage, starvation and high temperature (heat shock) also trigger a stress response dependent on a sigma factor called RpoH (σ^{32}). Among the genes controlled by σ^{32} is one that encodes GroE. This is a molecular chaperone that interacts with DNA polymerases IV and V (among many other proteins), protecting them from degradation by proteases and thus increasing mutagenesis.

There are three other mutagenic stress responses that are less well characterized. Two of them are specific for bacteria growing on solid media. One is called ROSE, an acronym for 'resting organisms in a structured environment'.[36] ROSE requires RecA and DNA polymerase I and it is independ-

[33] Hengge-Aronis, R. Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev*. 66, 373-395, 2002.

[34] Braeken, K., Moris, M., Daniels, R., Vanderleyden, J., Muller-Hill, B., Michiels, J. New horizons for (p)ppGpp in bacterial and plant physiology. *Trends Microbiol*. 14, 45-54, 2006.

[35] Wright, BE. A biochemical mechanism for nonrandom mutations and evolution. *J. Bacteriol* 182, 2993-3001, 2000.

[36] Taddei, F., Radman, M., Maynard-Smith, J., Toupance, B., Gouyon, P.H., Godelle, B. Role of mutator alleles in adaptive evolution. *Nature* 387, 700-702, 1997.

ent of DNA polymerase V and RpoS. Another one is called MAC ('mutagenesis in aging colonies') and it does not involve LexA, although it does require RpoS and DNA polymerase II.[37] A third response, the GASP phenotype[38] (growth advantage in stationary phase) relies on the SOS DNA polymerases II, IV and V and in an attenuated participation of RpoS. The GASP response allows survival of a small percentage of the bacterial population that consumes the debris of dying cells in long term batch cultures. Under these conditions, the birth and death rates are balanced. An increase in the mutation rate of cells in stationary phase is further supported by down regulation of the DNA repair pathways, some of which operate through intricate mechanisms that are highly energy consuming.[39]

The hypermutable state model

Hall proposed an additional argument to interpret the apparent directedness of adaptive mutations. It was what he called the hypermutable state model.[40] According to this model, although all non-growing bacterial cells in a selective medium are experiencing a stressful situation, only a minor subpopulation of them, perhaps between one in every 10^3 or 10^4 of cells enters a hypermutable state.[41] While in these circumstances, those bacteria that generate neutral or deleterious mutations die in a short time. However, if one of the mutations is a revertant that allows growth, the cell is relieved from the stress. It then proliferates exiting from the hypermutable state, building up just only growth-dependent mutations at a normal rate. Thus, the hypermutable state is transient. Eventually, the only cells that survive the stressful condition are those that never enter into the hypermutable state or those that do so and acquire a useful mutation. The fact that the frequency

[37] Bjedov, I., Tenaillon, O., Gerard, B., Souza, V., Denamur, E., Radman, M., Taddei, F., Matic, I. Stress-induced mutagenesis in bacteria. *Science* 300, 1404-1409, 2003.

[38] Finkel, S.E. Long term survival during stationary phase: evolution of the GASP phenotype. *Nature Rev. Microbiol*. 4, 113-120, 2006.

[39] Saint-Ruf, C., Pestut, J., Sopta, M., Matic, I. Causes and Consequences of DNA repair activity modulation during stationary phase in Escherichia coli. *Crit. Rev. Biochem. Molec. Biol.* 42, 259-270, 2007.

[40] Hall, B.G. Spontaneous point mutations that occur more often when they are advantageous than when they are neutral. *Genetics* 126, 5-16, 1990.

[41] Rosenberg, S.M. Evolving responsively: adaptive mutation. Nature Rev. *Genetics* 2, 504-515, 2001.

of mutations in selected revertants is notably higher than in the surviving cells that do not mutate the selected gene clearly satisfies the model.[42] It also adds evidence for selective capture rather than for selective generation.

The hypermutable state model has received support from Rosenberg's group.[43] According to these authors, the high mutation rate reaches its maximum with the coincident induction of the SOS and RpoS stress responses.

There are various mechanisms for adaptive mutations

Work in different laboratories has revealed that there are several ways by which bacteria can modify their genomes to relieve the selective pressure in a stressful environment. In other words, there are several types of adaptive mutations, each of them involving a molecular mechanism that sheds light into the seeming selectivity of mutation.

a) The episomal Lac system.[44]

As mentioned above, the *E. coli* FC40 strain carries a large conjugal plasmid which includes a fusion of the gene encoding the Lac repressor (*lacI*) with the *lacZ* gene encoding β-galactosidase. Therefore, it lacks the regulatory region of the operon and transcription starting from the promoter of *lacI* is constitutive. This construction is Lac^- because it carries a +1 frameshift in *lacI*, changing CCC to CCCC, although it is slightly leaky, conferring about 1% of wild type β-galactosidase level. The chromosome in the strain has a large deletion that encompasses the *lac* operon. When these cells are inoculated on solid minimal medium containing lactose as carbon source, colonies of Lac^+ mutants appear a few days later on the plate. In the absence of carbon source, Lac^+ mutations (as measured by subsequent plating on lactose) do not accumulate regardless the incubation time. Strain FC40 also reverts to Lac^+ during non-selected growth. In this case, mutations include duplication, deletions and large frameshifts,

[42] Drake, J.W. Too many mutants with multiple mutations. *Crit. Rev. Biochem. Mol. Biol*. 42, 247-258, 2007.

[43] Gallardo, R.S., Hastings, P.J., Rosenberg, S.M. Mutation as a stress response and the regulation of evolvability. *Crit. Rev. Biochem. Mol. Biol*. 42, 399-435, 2007.

[44] Foster, P.L. Stress-induced mutagenesis in bacteria. *Crit. Rev. Biochem. Mol. Biol*. 42, 373-397, 2007.

while mutations obtained during selection are almost exclusively -1 frameshifts. The latter are typically made by DNA polymerase IV (*dinB*), which is induced by the SOS and RpoS pathways. Adaptive mutations are severely reduced in GroE and polyphosphate kinase deficient cells, confirming the requirement for DNA polymerase IV. Under normal conditions, frameshift mutations are corrected by the mismatch repair system, which is insufficient or may be down regulated in stressed cells undergoing the transient hypermutation state. Mutants obtained under selection also differ from those arising during normal growth in that they require enzymes involved in the recombinational repair of double strand breaks, such as RecA, RecBCD and RevABC.

There are two models accounting for adaptive mutation in *E. coli* FC40 cells. One of them relies on the fact that the conjugal origin of the episome is subjected to continuous nicking. Occasional initiation of episomal replication at its vegetative origin is allowed by the energy provided by the leakiness of the Lac construction. Advancement of the replication fork towards the nick generates a double stranded break that is repaired by RecA, RecBCD and RuvABC recombination enzymes. Short patches of DNA synthesis required by this pathway are undertaken by the mutagenic DNA polymerase IV and by DNA polymerase II. This model accounts for the fact that the Lac construction needs to be in the episome in order to obtain adaptive revertants. A second mechanism leading to Lac^+ colonies of the FC40 strain consists in the 20-50 fold amplification of the *lac* locus.[45] These revertants appear somewhat later than the point mutants. Amplification does not require DNA polymerase IV or the other SOS-induced proteins, although it depends on RpoS, DNA polymerase I and the recombination proteins RecA, RecBCD and RuvABC. Interestingly, the amplified clones do not exhibit unrelated mutants as it is the case with the Lac^+ point mutants. Moreover, the Lac^+ phenotype of the amplified clones reverts to Lac^- upon re-plating in rich medium. Some investigators originally thought that amplification was an intermediate state in the formation of Lac^+ point mutants, but it was later shown that it consists on an alternative way to relieve the starvation stress by cells that never enter the hypermutation state.

[45] Hastings, P.J. Adaptive amplification. *Crit. Rev. Biochem. Mol. Biol*. 42, 285-311, 2007.

b) The transcription-dependent revertants of *trp* auxotrophs.

Amino acid starvation triggers the stringent response, which, as mentioned previously, up-regulates transcription of operons for amino acid biosynthesis. It has now been well established that transcription during prolonged starvation is mutagenic. The reason for this effect is that nucleotide bases are prone to undergo chemical modifications when present in single stranded DNA. For example, cytosine deaminates to uracil, which upon DNA replication, preferentially pairs with adenine instead of guanine. In turn, adenine spontaneously deaminates to hypoxanthine, which hydrogen bonds to cytosine rather than to thymine. In cells where the mismatch repair system is down regulated, these modifications remain in the DNA sequence.

Transcription generates localized single stranded structures in two ways.[36] One is the formation of a transcription bubble, where the DNA-RNA hybrid structure exposes the nontranscribed strand leaving it vulnerable to change. The other one, related to the negative supercoiling generated behind the transcription bubble, gives rise to stem-loop structures possessing susceptible unpaired bases. Since starvation for a particular amino acid specifically targets derepression of the corresponding operon, it is most likely that the adaptive missense mutations in the *trp* operon in Hall's studies are generated during transcription of this operon. This mechanism is coherent with the observed directedness of the revertant mutations.

c) Systems involving mobile genetic elements.

As mentioned previously, some adaptive mutations require either excision or insertion of DNA elements. Normally, molecular events of this kind are under tight control to avoid deleterious effects in the genome. However, stressful environments promote movements of such sequences,[46] providing the cells with an additional strategy for adaptation. For example, numerous studies have demonstrated that starvation elicits an increase in transposition frequency of mobile elements, which may be mediated by the RpoS or SOS responses. In the long term, this type of genome flexibility contributes to increase the genetic diversity of microbial populations.

[46] Shapiro, J.A. Genome organization, natural genetic engineering and adaptive mutation. *Trends Genet.* 13, 98-104, 1997.

Adaptive mutation and evolution

In proliferating bacterial populations, survival depends on efficient DNA replication, which requires high speed and fidelity. In contrast, a hostile environment where cells cannot multiply will favor the selection of mutants that are able to overcome the episode of crisis.

The basic difference between random and adaptive mutations is that the latter are beneficial by definition, since they increase fitness. Moreover, it has been observed that when adaptation requires more than one mutation, the appearance of the first one makes more expedite the production of those that follow. There are now three examples understood that illustrate this behavior: reversion of the *trp* double mutants, expression of the *ebg* operon and double reversion of *bgl* operon, all of them studied in Hall's laboratory. In each of these cases, reversion of the first mutation allows very slow growth. Then, selection operates to single out the second mutation which leads to rapid growth. This is undoubtedly a fine course of action for adaptation. No wonder evolutionary biologist Douglas Futuyma, excited by Hall's work on the evolution of the *ebg* operon to permit lactose metabolism, wrote: 'Thus, an entire system of lactose metabolism has evolved, consisting of changes in enzyme structure enabling hydrolysis of the substrate; alteration of a regulatory gene so that the enzyme can be synthesized in response to the substrate and the evolution of an enzyme reaction that induces the permease needed for the entry of the substrate. One could not wish for a better demonstration of the neo-Darwinian principle that mutation and natural selection in concert are the source of complex adaptation'.[47]

Common sense tells that the ability to accelerate variation in the genome offers a selective advantage for survival in a changing environment. Several studies, both theoretical and experimental, have confirmed this assertion. In this context, the hypermutation state could be particularly fitting because it increases the probability of obtaining an advantageous mutation when the majority of the cells undergoing a normal mutation rate do not produce it. A fine regulation of the hypermutation state lessens the likelihood of accumulating undesirable mutations.[48] First, it is transient,

[47] Futuyma, D.J. Evolution (Sunderland, M.A.: Sinauer Associates), pp. 477-478, 1986, cited by Miller, K.R. in *Finding Darwin's God*. Perennial, Harper Collins Publishers 2002.

[48] Foster, P.L. Adaptive mutation: implications for evolution. *BioEssays* 22, 1067-1074, 2000.

i.e., when adaptation to the medium is achieved, a return to low mutation rates is selected for. But also, it is restricted to space, as it is clearly exemplified by mutations induced by double stranded breaks, transcription of defined operons and movement of genetic elements. In spite of the clear advantages of confining mutation in space and time, there are occasions in which adapted mutants maintain a mutator phenotype. This outcome is thought to result from adaptive mutations originated in strains with a mutator allele, a property that would be transmitted by hitchhiking in conjunction with the favorable alleles they produce.[49]

It would be very difficult to establish the precise contributions of growth dependent mutations, adaptive mutations and horizontal gene transfer to bacterial evolution. This problem could perhaps be approached experimentally, although laboratory studies are generally short term, whereas microorganisms in their natural environments confront long periods of starvation. Having this limitation in mind, it is worthwhile to highlight recent results obtained by Yeiser *et al.*[50] with bacteria struggling to survive in stationary phase. These investigators confirmed that SOS-induced DNA polymerases II, IV and V enhance long-term survival and evolutionary fitness of bacteria under stress. When grown individually, wild-type and SOS DNA polymerase mutants exhibit similar cell yields and stationary phase survival patterns. However, when the wild type and the mutant strains are co-cultured and must therefore compete for nutrients, SOS polymerase mutants undergo a marked reduction in fitness and fail to express the 'growth advantage in stationary phase phenotype' (GASP). Since DNA polymerase V is the most mutagenic, it is remarkable that mutants of this enzyme are the most affected in the competition experiments. According to these authors, DNA polymerase V may provide the mutational raw material for natural selection in a manner superficially similar to the increase fitness accompanying the absence of the mismatch repair system.

[49] Kivisaar, M. Stationary phase mutagénesis: mechanisms that accelerate adaptation of microbial populations under environmental stress. *Environ. Microbiol*. 5, 814-827, 2003.

[50] Yeiser, B., Pepper, E.D., Goodman, M.F., Finkel, S.E. SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness. P*roc. Natl. Acad. Sci. USA* 99, 8737-8741, 2002.

Concluding remarks

Unraveling the adaptive mutation phenomenon has allowed us to become aware that the complexity of living organisms is not the outcome of a sole random mutational process, as it is most commonly regarded. Instead, it has become clear that throughout evolution there have also been adaptive mutations stimulated by a variety of fine feedback mechanisms. These include activation of error prone DNA polymerases, down-regulation of DNA repair enzymes, gene amplification, movement of mobile genetic elements, development of a transient hypermutation state in some cells, localization of mutations in genomic space to minimize deleterious mutants, various types of recombination events, etc.

It is most likely that these induced mutations have had a key role in determining bacterial evolution, since natural habitats are often stressful due to a lack of nutrients or some other unfriendly condition. There is still a third kind of gene variation that is widespread in the microbial world and has played a decisive role in bacterial evolution, namely, horizontal gene transfer. In spite of its importance, however, the description of this phenomenon goes beyond the scope of this essay.