© N. Babudri*,
H. Lancioni, A. Achilli

Dipartimento di Biologia Cellulare e Ambientale, University of Perugia (Italy)

ENVIRONMENTAL STRESS AND MUTAGENESIS IN ENTERIC AND NON-ENTERIC BACTERIA

❀ **Mutations are fundamental for evolution. For many years it has been thought that mutagenesis occurs only in dividing cells. Now it is clear that mutations arise in non-dividing or slowly dividing microorganisms. Natural populations spend most of the time in stressful environments where their growth rate is highly reduced. Thus, the existence of a mutagenesis process, independent of multiplication (stress-induced mutagenesis, SIM), might have a profound evolutionary role. In the presented paper we review the state-of-the-art in enteric and non-enteric bacteria. We describe different experimental systems as well as the mechanisms and models presented to explain the huge amount of data obtained in more than twenty years of research.**

❀ **Key words:** stress-induced mutagenesis; enteric and non-enteric bacteria; evolution.

INTRODUCTION

Since the mid-1950s, microbiologists have been aware of mutations occurring in non-dividing or slowly dividing populations of cells [1]. The idea that mutations are random events which form only in dividing bacteria, as shown by Luria and Delbruck in their pivotal experiments [2], was prominent among researchers until 1988. At that time, Cairns and coworkers [3] challenged this dogma. In their paper "The origin of mutants" they confirmed the previous studies [1] showing that mutations can arise in apparently static bacterial populations of *Escherichia coli* when subjected to non-lethal selective pressure. They suggested that only selected mutations, not deleterious or neutral ones, appear in the population under selective pressure (adaptive mutagenesis). In the beginning, adaptability was interpreted in the strict sense, with the suggestion that exclusively selected, beneficial mutations (directed mutations) might arise. Later, experimental evidence supported the idea that other, unselected, mutations may arise along with the beneficial ones [4,5,6]. Nowadays the term adaptive mutagenesis in *E. coli* is used in a broader sense, including selected as well as unselected mutations [7]. Different terms have been used besides "adaptive mutation" [8]. For the purpose of the present review we will call it stress-induced mutagenesis (SIM).

One fundamental question is the evolutionary significance of SIM: is it a trait selected by evolution or is it a byproduct of a stress inducible function that was designed to counteract the stress itself? Most mutations are deleterious. Therefore, the evolvability of genetically controlled pathways generating mutations as a response to environmental stress seems rather wasteful. Some researchers consider SIM and the consequent increase in mutation rates as an adaptive strategy that could enhance variability, and consequently the chance of evolution of the bacterial population under stressful selective pressure [9, 10]. Other authors regard SIM as a by-product of processes which enhance survival during stress [11]. Both camps could be right: different systems could operate in nature to shape mutation rates in a stressful condition [12]. Here we will provide a brief overview of the experimental systems, mechanisms and models of SIM.

The review does not exhaust the vast literature on the topic; for more information and opinions we refer the reader to the following excellent reviews [7–10, 13].

Stress-induced mutagenesis in E. coli: the Lac$^+$ reversion assay

LAC$^+$ POINT MUTATIONS AND AMPLIFICATIONS IN THE LAC$^+$ REVERSION ASSAY

In the Lac assay the *E. coli* FC40 strain is used. The FC40 strain is unable to use lactose as the sole carbon source (Lac$^-$) because of a deletion of the chromosomal *lac* operon and because of a *lacI-lacZ* fusion gene with a +1 frameshift mutation in *lacI* in a F'conjugative plasmid. The strain is pre-grown on a medium with glucose

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and without lactose and then spread on lactose plates on which only Lac$^+$ cells can form colonies [14]. Generation-dependent Lac$^+$ mutants that arise before plating on lactose medium form visible colonies after two days of incubation. Lac$^+$ colonies that arise on selection plates accumulate from day three on. The stress-induced Lac$^+$ colonies result from at least two independent mechanisms: point mutations and amplification of the *lac* region on the F' 128 plasmid [15]. The point minus one frameshift mutations dominate during the first week of incubation. It is worth mentioning that mutagenesis during logarithmic growth includes duplications and larger frameshifts in addition to deletions. Tandem repeats containing 20–50 copies of the *lac* region are found in approximately 40% of Lac$^+$ colonies at day eight of incubation and their percentage increases thereafter [15–19]. The frameshift *lac* allele encodes for a barely active protein, but its amplification leads to the production of enough ß-galactosidase activity to restore growth on the selective plates. Thus, Lac$^+$ colonies are formed via two independent mechanisms: point mutations and amplification of the leaky *lac* allele. The *lac*-amplified colonies appear later than the point mutants and can be distinguished from point-mutant colonies based on their unstable Lac$^+$ phenotype; upon re-plating on rich medium containing a color indicator for ß-galactosidase activity, *lac*-amplified cells give rise to both white (Lac$^-$) and sectored colonies (blue with white sectors). White colonies /sectors are composed by cells where, in the absence of selection, the *lac* allele returned to the wild type copy number (deamplification) [20].

Non-selected markers and the hypermutable subpopulation

Torkelson et al. [21] tested the possibility that random, genome-wide mutations occur in a small subpopulation of cells exposed to selection as proposed by other authors [22, 23]. Non-selected mutations were scored among Lac$^+$ revertant colonies, Lac$^-$ colonies not exposed to selection (Lac$^-$ unstressed colonies) and Lac$^-$ colonies exposed to selection (Lac$^-$ stressed colonies from the same plates where Lac$^+$ revertant clones were selected). Three replicons of the cells, namely a pBR322-derived plasmid, the F' 128 plasmid and the bacterial chromosome, were considered. Colonies which have reverted to Lac$^+$ under stress mutated at higher frequency with respect to Lac$^-$ unstressed and stressed colonies, respectively, in all three replicons. This implies that SIM in the FC 40 assay is not specifically targeted to the lac allele. Moreover, since hypermutagenesis was seen among Lac$^+$ revertants but not among Lac$^-$cells from the same starved cultures, this implies that a subpopulation of cells exposed to starvation on lactose experience a genome-wide hypermutable condition. A hypermutable condition could be the consequence of heritable defects in error-free repair systems like mismatch repair (MMR), the post-replicational error-correction system and the proof-reading activity of DNA polymerases. Bacterial mutants with constitutively high mutations rates (constitutive mutators) could form as a consequence of the defects in these repair systems. Torkelson et al. [21] tested Lac$^+$ revertants with the associated unselected mutations for constitutive mutator phenotype, showing that most of them were not constitutive mutators. The authors also provided proof that the majority of unselected mutations associated with a Lac$^+$ reversion formed coincidentally with it and not during the growth of the Lac$^+$ colony. Then the hypermutable state is a transient condition acquired during exposure to the selective environment.

The genetic requirements of stress-induced mutations

We briefly review the genetic requirements of stress-induced point mutagenesis and amplifications which are more relevant for the understanding of the models described below. i) The stress-induced point mutagenesis in *E. coli* FC 40 requires proteins involved in the repair of Double Strand Breaks (DSBs) via homologous recombination, that is RecA, RecBCD, RuvABC. Stress-induced mutants are reduced but revertants during growth form at a normal rate when these genes are inactivated [13, 24–27]. ii) The SOS system is also required. The SOS response is the DNA repair system described in *E. coli* induced upon treatment of bacteria with DNA damaging agents that arrest DNA replication and cell division (for a review on SOS repair see reference # 28). The primary task of the SOS response is to restart replication before the cells die after exposure to DNA damaging agents. However, the SOS response can also be induced in aging colonies and when cells reach saturation in rich medium [29], suggesting its possible role in the response of cells to various stresses. This system is regulated by both the LexA transcriptional repressor and the RecA recombinase. There are more than 40 genes known to be involved in SOS repair. Most of these genes are induced rapidly and are involved in error-free DNA repair, including base excision repair, nucleotide excision repair, and recombinational DNA repair. If the DNA damage levels are high, the mutagenic phase of SOS is triggered. This phase of the SOS response is mediated by DNA polymerases that replicate past template lesions, in a process called translesion DNA synthesis, or TLS. There are three SOS-induced DNA polymerases in *E. coli*, Pols II (B-family), IV and V (both Y-family, specialized, error-prone polymerases), and all are engaged in various aspects of TLS. PolII is the only one that possesses a 3'-5'exonuclease proofreading activity and it is the least error-prone. PolII can bypass some DNA lesions and can extend DNA termini when lesions are bypassed by other polymerases. Pol V is encoded by the *umuDC* operon and carries out TLS across pyrimidine dimers and other types of bulky DNA lesions. It is error-prone on both damaged and undamaged DNA. Pol IV is encoded by DinB and bypasses a more limited range of lesions in comparison to PolV. It is able to bypass some guanine adducts such as, for example, benzo (a)pyrene guanine adducts

[30] at high fidelity, and it incorporates adenine opposite the 3'-thymine of a UV light-induced TT (6–4) photoproduct; however, it is error-prone on undamaged DNA, thus causing untargeted mutagenesis [31]. Formation of 85% stress-induced point mutations leading to Lac^+ phenotype requires Pol IV, in contrast to mutagenesis in dividing cells [9] and the sole role of the SOS response in stress-induced mutagenesis seems to be the up-regulation of PolIV [32]. iii) SIM is strictly dependent on stress response in *E.coli* FC40. This is documented by its requirement of the RpoS gene product [33]. RpoS (σ^{38}) is the master gene of the general stress response activated in *E. coli* cells upon entry into the stationary phase or when they are subjected to other stresses (growth-rate reduction, high osmotic pressure, low pH, extreme temperatures) [34]. Sigma factors are the subunits of RNA polymerase that allow cells to direct transcription to specific promoters. During exponential growth most genes are transcribed by RNA polymerase containing $\sigma^{70.}$ As cells enter the stationary phase the amount of active RpoS increases and genes required for survival under stressful conditions are transcribed. In *E.coli*, hundreds of genes are known to be controlled by RpoS [35]. RpoS is also required to transcriptionally up-regulate Pol IV, thus linking environmental stress and error-prone TLS [33]. iv) MMR is a conserved repair system that repairs mispaired bases and one or a few nucleotide insertion /deletion heteroduplexes arising during replication or from other sources [36]. MMR was first linked to SIM in *E.coli* FC40 cells because of the observation that Lac^+ mutants were almost all −1 deletions in a region of small nucleotide repeats. This mutation spectrum is different from growth-dependent reversions [37, 38] but it is identical to growth-dependent reversions in MMR defective strains [39]. Thus it has been proposed that a limiting MMR could be responsible for Lac^+ revertants formation and efforts have been made to clarify this point. Interestingly, it was shown that the function of the MMR system becomes limiting transiently during stress-induced Lac^+ mutagenesis via a limitation in functional MutL [40, 41]. The mechanism of MutL functional limitation is not understood [9]. It has been observed that MutL protein levels do not decrease in most starving cells though they might in a hypermutable cell subpopulation, giving rise to stress-induced point mutants [40, 42]. v) The position of the genes (chromosomal / episomal) was also considered to be relevant for SIM. The high level of adaptive reversion to Lac^+ seen in FC40 requires wild-type conjugal functions and that the lac allele is present in the episome [43, 44]. Foster and Trimarchi [43] demonstrated that E. coli strains with mutations in two genes that affect conjugation (traD and traQ) show a reduced reversion rate to Lac^+ with respect to the wild-type strain. The most likely role for the conjugal functions is to initiate recombination by producing a nick at the conjugal origin. Recently, Shee et al. [45] developed a mutation assay for the reversion of a chromosomal tetA+1 frameshift allele in starving F^- cells, depending on the induction of the endonuclese I-SceI with consequent DSBs formation. When tetA+1 cells acquire a compensatory frameshift mutation in the gene, cells become tetracycline resistant. The authors provided proof that DSBs-dependent SIM (see below) causes half of spontaneous chromosomal frameshift and base substitutions mutations in starving plasmid-free cells. They also discuss the possibility that tetracycline resistance can be due to amplification of the *tetA+1* allele, independently of stress; however, they consider this an unlikely event in their system. We refer the reader to the original paper for more details [45]. Shee and colleagues [46] demonstrated that SIM is approximately 100 times less active on the bacterial chromosome with respect to F'. This is probably because DSBs-dependent SIM needs a homologue partner molecule to operate, which could be a duplicated chromosomal segment or a sister chromosome. vi) The formation of *lac*-amplified cells has genetic requirements which only partially overlap to those of point mutations. Indeed, it is controlled by the RpoS gene product [47] and it requires RecA, RecBCD and RuvABC [19] like point mutations. However, unlike point mutants, amplified revertants do not require polIV [48]. Finally, *lac*-amplified cells do not show genome-wide mutagenesis. Thus, the available data suggest that point mutants and amplified mutants result from distinct responses of *E. coli* FC 40 cells to stress. For more information on the proposed molecular mechanisms of *lac*-amplified cell formation see Galhardo et al. [9].

The Double-strand breaks (DSBs)-dependent stress-induced mutagenesis model in E.coli FC40 and other bacteria

E. coli FC40

According to the DSBs-dependent SIM model [9], the initiating event for point mutations is the production of a DSB in the episome. Frost and Manchak [49] showed that cleavage at oriT of the F^+ plasmid occurred also during the stationary phase. The authors propose that this could account for the higher propensity of F^+ cells to undergo SIM with respect to F^- cells in the Lac^+ reversion assay. While the Lac^- cells are on lactose, the leaky lac allele provides enough energy to allow replication to be occasionally initiated from the episome's vegetative origins. When the moving replication fork encounters the persistent nick at oriT, a double strand end is produced and this initiates DSBs repair. This recA and RecBCD-dependent repair proceeds via strand invasion of either the homologous duplicated portion of the same episome or of another copy of the same episome. New DNA is then synthesized. Recent data [50] suggest that repair DNA polymerases I, II and III compete with DNA Pol IV for the primer terminus during the acts of DSBs. Up-regulation of Pol IV by RpoS could tilt the competition in favor of error-prone Pol IV at the expense of more accurate polymerases, thus producing stress-induced mutations. The Holiday junction produced during recombination is re-

solved by the RuvAB / RuvC enzymes (for a schematic view of the process see Galhardo et al. [9]). If the region of newly synthesized DNA includes the *lac* region then Lac$^+$ mutations may arise; if other regions of the episome are included, then the genes in those regions may accumulate mutations. While the recombination-dependent mechanism occurs at a high rate on the episome, it can also produce mutations on any replicon, including the chromosome, whenever the replication fork encounters a nick, or when a DSB is produced. Secondary mutations occur in this case. To explain the formation of a transient hypermutable subpopulation within a stressed population Galhardo et al. [9] proposed the following model. During growth, spontaneous SOS induction occurs in about 1% of cells because of DSBs, with cells cycling in and out of the SOS-induced population [51]. Repair of DSBs with SOS induction is not sufficient to cause stress-associated mutagenesis and induction of the RpoS response is also required. The SOS system and RpoS induction could lead to the overexpression of PolIV which, together with loss of MMR, could cause a fraction of stressed cells to become hypermutable (Figure 1). The genetic requirements of DSBs SIM in *E. coli* FC40 are shared by other bacterial species in different experimental systems

Ciprofloxacin resistance in E. coli

Ciprofloxacin is an antibiotic of the quinolone class which targets gyrase and topoisomerase IV, enzymes that coil and uncoil DNA and are fundamental in DNA repair processes. The key event in the quinolone action is reversible trapping of gyrase-DNA and topoisomerase IV-DNA complexes with a consequent bactericidal effect [52]. Interestingly, when *E. coli* cells are plated on a bacteriostatic dose of ciprofloxacin, the population that survives does not grow but it accumulates resistant mutants over the course of two weeks. This phenomenon is very similar to the accumulation of Lac$^+$ revertants in the FC40 assay [53]. The ciprofloxacin stress-induced resistance requires Pol II, and Pol IV (DinB) and the DSBs repair genes (SOS-regulated RecA, RuvA, RuvB) [9] like *E. coli* FC40 but also requires PolV (UmuDC). Thus, a mutagenesis pathway is induced in cells stressed by ciprofloxacin; this pathway causes the adaptation of the bacterial population to the stress itself.

Stress-induced ß-lactam antibiotic resistance in E. coli chromosome

Petrosino et al. [54, 55] developed a system which allows study of ß-lactam antibiotic resistance due to mutations in

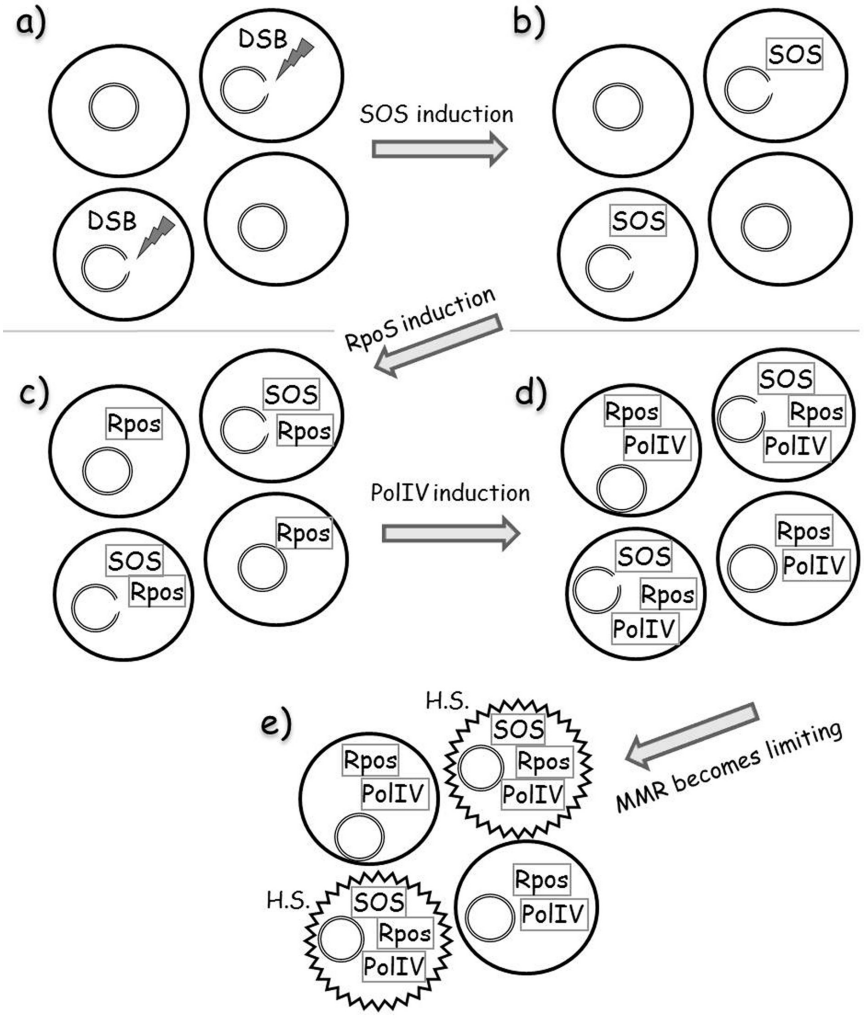

Fig. 1. DSBs may form in some cells during growth (a) triggering the induction of the SOS system (b). Upon entry into the stationary (non-growing) phase, RpoS is induced as a consequence of nutritional stress. Thus, the SOS and RpoS responses are both induced in some cells (c). RpoS activity up-regulates DinB transcriptionally, thus licensing polIV for error-prone DSBs repair via homologous recombination (for a schematic view of the molecular process see Galhardo and coll., reference #9) (d). The large number of PolIV errors may cause MMR to become limiting making a fraction of the population transiently hypermutable, H.S. = hypermutable subpopulation (e)

the *ampD* gene in the same conditions that provoke stress-induced mutagenesis in the FC40 system (lactose as a sole carbon source for Lac⁻ cells). The assay allows the detection of forward mutation (both base substitutions and frameshift) at the *ampD* locus. The authors demonstrated that -lactam resistance requires DSBs repair proteins, the SOS and the RpoS stress responses and pol IV, like *lac* reversions. In addition, it also partially requires PolV. Therefore, ß-lactam resistance is induced via DSBs repair-associated mutagenesis. Interestingly, the authors showed that the number of stress-induced base substitutions, which was not detectable in the original FC 40 assay, was higher with respect to frameshift mutations as shown in other systems [55, 56]. These findings have an important evolutionary implication: stress can not only induce loss-of-function mutations but also change-of-function mutations, which means that modifications of gene functions might occur as a consequence of exposure to natural stress factors.

Non-enteric bacteria: Bacillus subtilis and Pseudomonas putida

As a model system for studying stress-induced mutagenesis, *B.subtilis* is particularly intriguing. It is a paradigm for prokaryotic differentiation which occurs during post-exponential growth, leading cells to the ability to bind as well as take up and incorporate DNA from the environment (competence for transformation). Only those cells in which the ComK transcriptional activator accumulates at a threshold level become competent (10% of the total population). In 2002 Sung and Yasbin [57] hypothesized that SIM could be related to differentiation of *B.subtilis* into competent cells. They developed a simple experimental system in which the reversion frequencies to prototrophy in a strain auxotrophic for histidine, methionine and leucine starved for these amino acids are studied and provided the first evidence of stress-induced mutagenesis in *B.subtilis*. In *B.subtilis,* stress-induced mutagenesis is controlled by the *comA* and *comB* genes coding for transcription factors, which regulate early and late phases of competence, respectively [58]. This finding is the first proof of a link between stress-induced mutagenesis and differentiation in bacteria. The involvement of the Com genes, the fact that about 1% of the revertants carried a second, non-selected mutation, and the fact that in *B. subtilis* stressed cells the MMR system is limiting [58] support the idea that, as in *E. coli* FC40, the population under stress includes a hypermutable subpopulation. In contrast to *E. coli* FC40, stress-induced mutagenesis in *B. subtilis* does not require either recombination or SOS functions but requires a functional *yqjH*, a gene which encodes ortholog of Pol IV of *B.subtilis* [59]. Vidales et al. [60] and Debora et al. [61] provided proof on the importance of oxidative damage in stress-induced mutagenesis *B. subtilis*, a DNA insult whose role is controversial in stress-induced mutagenesis in *E. coli* [62–64]. Oxidative stress-promoted lesions, like those induced by the hydroxyl radicals, are among the most prominent DNA modifications within cells, generating 8-oxo-G which causes transversions. Both 8-oxo-GTP and 8-oxo-dGTP may also be produced in the deoxynucleoside triphosphate and nucleoside triphosphate pools with subsequent incorporation into DNA. *B. subtilis* relies on the glycosylases MutM and MutY to contend with the potential mutagenic effects of 8-oxo-G: A mispairing and on YtkD and MutT proteins to prevent the genotoxic effects of oxidized precursors. Vidales et al. [60] showed that stress-associated mutagenesis is potentiated in starved *B. subtilis* cells lacking YtkD, MutM and Mut Y proteins, thus showing the importance of oxidative stress in generating stress-induced mutations. Recently, the impact of uracil in DNA on stress-induced mutagenesis was studied by López-Olmos [65] who showed that the accumulation of mutation in chromosomal genes in *B. subtilis* non-dividing cells was significantly diminished following the disruption of two genes involved in the removal of uracil and hypoxanthine from DNA, that is the *ung* gene coding for uracil DNA-glycosylase and the *ywqL* gene coding for a putative endonuclease V homolog. These findings suggest that under nutritional stress uracil which is the result of deamination of cytosine can be removed in an error-prone manner promoting mutagenesis. Stress-induced mutagenesis has also been studied in *P. putida*, a bacterium belonging to the genus *Pseudomonas* which is widely distributed in different environments. The *P. putida* experimental system is based on the selection of phenol-degrading mutants due to the activation of a silent phenol monooxygenase gene *pheA* on a plasmid under carbon starvation on minimal agar plates containing phenol as the only carbon source [66]. It is worth mentioning that during the incubation on phenol, the number of viable cells detected by plating was constant but staining for viable cells showed that many cells died. Therefore, the population resembles those in a long-term stationary phase where subpopulations better adapted to the new environment overgrow the original one (growth advantage in stationary phase, GASP) [9, 67]. Because of this peculiarity and because *P. putida* differs from *E. coli* in several aspects of DNA repair and damage tolerance mechanisms, it could offer an opportunity, together with *B. subtilis*, of expanding the view of stress-associated mutagenesis beyond the border of *E. coli* and *S. enterica* systems.

THE SELECTION-ONLY MODEL

In 2003, Roth et al. [68] published a paper in which they theoretically examined the hypermutable state model and concluded that it is conceivable that a heavy genetic burden of lethal mutations is associated with reversions to Lac⁺. Thus, the authors claimed, it is unlikely that the revertants would survive in the long term and that the system has been selected by evolution because of its property of enhancing

mutagenesis. Rather it enhances survival under stressful conditions. Later, the studies of Andersson et al. [69] using a Lac- Salmonella typhimurium strain containing the F' lac plasmid of E. coli FC40, lead the authors to propose a model in which selection is the main evolutionary force that determines the accumulation of mutants in populations under stress. In this model, no increase in mutation rates is proposed to account for the rapid adaptation of cells in a growth-limiting environment. The model is as follows: during the log phase, variants with a small increase in the gene product necessary to overcome the stress can form because of amplifications (or other events such as duplications and leaky point mutations). These variants can grow slowly on the selective medium. Additional mutations that improve growth rate can occur during cycles of divisions. Indeed, amplification provides more mutational targets. Then the new mutants can overgrow the colony. The selection-only model has been proven to be true in two systems which are described in the following paragraphs.

S. typhimurium: the case of protamine resistance and small colony variants reversion

This is an interesting example of the possible role of amplification in evolution. Therefore, we included it in the review although it is about replicating cells.

Antibiotic resistance is often associated with a reduced growth rate of the resistant cells [70]; however, bacteria can acquire second-site mutations that abolish the growth defect. Pränting and Andersson [71] exploited how slow-growth variants (small colony variants, SCV) of *S. typhimurium* escape from the growth reduction caused by a mutation in the *hemC* gene, conferring resistance to the antimicrobial peptide protamine. The mechanism of protamine resistance is complex and not completely understood. The initial step is thought to be an electrostatic interaction between the positively charged protamine and negative charges on the bacterial membrane [72]. Mutations in the gene *hemC*, encoding porphobilinogen deaminase, an intermediate in heme biosynthesis, confers resistance to protamine but, at the same time, causes a slow-growth phenotype. The slow growth phenotype of a protamine-resistant (*hemC*) mutant is unstable and cells can revert to a faster-growing type [72]. Pränting and Andersson [71] showed that a faster growth is restored via a multi-step process where fitness is gradually increased in SCV. On the basis of their results they proposed the following model. The level of the enzyme coded by the defective allele *hemC* is increased in cells with *hem C* amplifications, which are common in growing cells, and cause a small increase in growth rate. The amplifications increase the probability of point mutations because they increase the number of targets. Point mutations restoring the function and increasing fitness will occur. Amplifications are counterselected in those cells with favorable point mutations leaving those cells with only one copy of the *hemC* gene and a further fitness increase. Thus, the experiments of Pränting and Andersson [72] not only confirm the amplification models but also show all the intermediate steps of the model, in slowly replicating cells.

Stress-induced mutagenesis in S. typhimurium: the pur system

Yang et al. [73] devised an experimental setup to study stress-induced mutagenesis on lactose plates in *S. typhimurium,* which is naturally Lac- (the Pur-Lac system). The *S. typhimurium* purR gene encodes a transcriptional repressor regulating gene expression of de novo purine nucleotide biosynthesis. It represses purD gene transcription by binding to the 16-base pair purD operator. *E. coli* lac genes were genetically engineered into the *S. typhimurium* chromosome and repressed by purR super-repressor [purR (s)] so that they could be used as an indicator of adaptive mutations in purR (s) or in the purD operator. Yang et al. [73] observed that mutations in purR (s) or in the purD operator accumulated with the characteristics of stress-induced mutagenesis when the mutant strain was placed on a minimal lactose plate, thus allowing the reversion from the Lac- to the Lac+ phenotype. Importantly, mutational targets (*purR* /*purD*) are placed on the main chromosome not on an episome. Recently, the system was used by Quiñones-Soto and Roth [74] to test the selection-only model. The authors provided the proof that Lac+ revertant colonies are initiated by the appearance of leaky Lac+ mutants prior to selection (not amplifications). The Lac+ phenotype is improved further because of a secondary mutation acquired during growth under selection. No evidence of genome-wide mutagenesis was obtained; thus amplification, selection and a normal rate of mutagenesis are sufficient to account for mutants formation in this system. However, the authors pointed out that an important question still has to be answered; is the behavior of the Pur-Lac system fully explained by selection alone? They address this complex point in the discussion of their paper [74]. Here, we just want to underline the idea that all systems used to study SIM are never simple and that this could be related to the vast array of solutions that cells have evolved to cope with stress.

STRESS-INDUCED MUTAGENESIS IN NATURAL E.COLI POPULATIONS

In 2003, Bjedov et al. [75] published a paper which received much attention because, according to the authors, it demonstrated that about 80% out of 787 *E. coli* natural isolates from a wide range of habitats showed stress-induced (ageing-associated) mutagenesis requiring the RpoS-controlled stress response and some functions of other stress-induced mutagenesis systems. To estimate mutagenesis in aging colonies, Bjedov et al. [75] measured the frequency of mutations conferring resistance to rifampicin (Rif^R) in one-day and seven-day old colonies of 787 worldwide natural isolates from different ecological niches. They plated cells from overnight cultures on filters laid on rich medium plates which were incubated at

37 °C for one to seven days. Bacterial colonies were resuspended in 1 ml of media and incubated for one hour at 37 °C to allow for antibiotic resistance expression. Then appropriate dilutions were spread on antibiotic plates. The antibiotic resistant mutants were scored and the frequency of mutation was calculated. The median value of the frequency of Rif^R mutants were 5.8×10^{-9} on day 1 and 4.03×10^{-8} on day 7 for all strains. Thus, the frequency of Rif^R mutants increased on average seven-fold between day 1 and day 7. Moreover, Bjedov and collaborators [75] showed that the extent to which Rif^R mutants accumulated during aging varied from one *E. coli* isolate to another and was dependent on RpoS. These data convinced the researchers that stress-induced mutagenesis in natural bacterial population is widespread and selected by evolution as a strategy to improve survival under different environmental stresses (reviewed in [9]).

Later, Wrande and coll. [76] published a paper showing that accumulation of Rif^R mutants in aging colonies of E. coli is due to growth under the selection of Rif^R mutants, not due to stress-induced mutagenesis. In their experimental setup, colonies were started by spotting a few cells on filters on rich, non selective medium. During the first two days the colony populations increased to 10^{10} cells and then stopped as previously reported by Bjedov and collaborators [75]. Since the colony was started with few cells no Rif^R mutant was present in the initial inoculums. Thus, any accumulation of Rif^R mutant observed in experiments protracted for several days might have been due to Rif^R formed either on rich medium plates or on selective plates. The data obtained by Wrande and coworkers [76] lead the authors to conclude that Rif^R mutant cells arose on rich medium where they formed clones selected during incubation on rifampicin plates, thus supporting a model where selection and not mutagenesis is the driving force during aging. Their conclusions were based on the following results: i) they observed that the frequency of Rif^R mutants increased exponentially during the aging period, while a linear increase is expected if mutants arise by mutagenesis of a non-growing population. ii) The authors reasoned that if the increase in the frequency of Rif^R mutants with aging was due to few mutant cells arisen during growth (day 1–2) on rich medium, then the mutant cells should have formed localized clones (papillae) at few sites within the aging colonies. In contrast, if aging induced mutagenesis in a non-growing cell population (by day 2 and on), individual Rif^R cells should have arisen independently, broadly distributed throughout the aged colony, without forming papillae. Aged colonies were divided into 16 sectors and each sector was assayed individually for Rif^R mutants. First, mutant cells were found to be positioned within papillae in the aging colony, consistent with the growth of the mutant population from individual precursor cells. Second, mutant cells within a single sector of the colony carried the same sequence alteration, demonstrating their clonal relatedness. Third, the Rif^R mutants that accumulated during aging showed no increase in the frequency of secondary mutations, suggesting that they were not made by induction of genome-wide mutagenesis. Fourth, the growth advantage of Rif^R mutants during aging could be demonstrated in reconstruction experiments. The data published by Wrande and coll. [76] shed strong doubts on the conclusions of Bjedov et al. [75]; therefore, in our opinion, at present there is no solid proof that stress-induced mutagenesis in natural *E. coli* isolates is a general phenomenon.

TRANSCRIPTION–ASSOCIATED MUTAGENESIS

Spatial restriction of SIM within genomes could be viewed as a system to direct mutations to specific sites limiting their possible detrimental effects on cell fitness. In this respect, transcription-associated mutagenesis can possibly be exploited by evolution. Wright et al. [77] found that the reversion rate of the *leuB* allele correlates with the expression level of the gene in different conditions. If the hypothesis of transcriptional-associated mutagenesis in stressed bacterial cells is correct, one would expect stress-associated mutagenesis to be influenced by the absence of factors coupling transcription with mutagenesis. Bridges [78] found normal stress-associated mutagenesis in an *E.coli* strain defective in the transcription repair coupling factor Mfd. Later, Barinovi and collaborators [79] showed that transcription of the *trpB* allele in *E. coli* is not mutagenic under conditions of tryptophan starvation, thus showing that SIM is independent of transcription. However, more recently, Cohen and Walker [80] provided evidence that the elongation factor NusA, which interacts with the core component of RNA polymerase [81], is required for SIM in *E.coli* FC40. They showed that FC40 cells carrying the temperature-sensitive *nusA11* allele display an approximately 470-fold reduction of the rate of reversion to Lac^+ and have stress-induced tetracycline resistance mutations abolished at the permissive temperature (30 °C). On the basis of these and other results, the authors suggested that NusA may play a role in SIM, providing a link between transcription and mutation formation [80]. To test the possible involvement of transcription in stress-induced mutagenesis in a $Gram^+$ species, Ross et al. [82] constructed a *B. subtilis* strain defective in the *Mfd* gene, which codes for a protein that couples DNA nucleotide excision repair to transcription, causing preferential repair of actively transcribed genes. In this strain, it might be expected that stress-associated mutagenesis would be increased because of a lack of efficient repair of lesions in actively transcribed genes. Contrary to this, Ross and coll. [82] showed that the *Mfd*-defective strain mutated significantly less than the wild-type strain when incubated on media selecting for prototroph revertants. In the discussion, the authors [82] pointed out that their results were not expected on the basis of what is known about the role of Mfd. They suggested the possibility that Mfd mediates mutagenic processes through various mechanisms; this is discussed in deeper detail in their paper. In *B. subtilis*, Pybus et al. [83] placed the allele *leuC427* (point mutation)

under the control of an isopropyl-ß-D-thiogalactopyranoside inducible promoter and tested the frequency of reversion to leucine prototrophy under conditions of transcriptional induction and repression in starved cells. Their results demonstrate that the level of reversion to leucine prototrophy increased markedly in parallel with the induced increase in transcription. Therefore, it has been established that SIM is coupled with transcription in *B. subtilis*: however, the exact molecular mechanism is yet to be elucidated. A model for transcription associated mutagenesis has been proposed by Wright [84] who suggested that transcription would induce local modifications in DNA supercoling thus facilitating the formation of unpaired bases with a consequent increase in mutagenesis in specific sites. Wright [84] also suggested that glucose starvation could induce non-random mutagenesis directed toward derepressed genes, an observation particularly intriguing since glucose starvation is frequent in nature. Another way in which mutations could be targeted to specific genes is transcriptional mutagenesis. Transcriptional mutagenesis results from the bypass of base damages by RNA polymerases in an error-prone manner. It has been documented in several systems in vitro and vivo, in prokaryotic and eukaryotic cells. For example, it has recently been shown by Clauson et al. [85] that in *E. coli* RNA polymerase is able to bypass AP sites (including those formed during repair of non-bulky lesions by base excision repair) as well as strand breaks causing transcriptional mutagenesis via adenine incorporation. As a consequence of transcriptional mutagenesis, mutant mRNAs and the correspondent mutant proteins may form. Transcriptional mutagenesis might be relevant to adaptive mutagenesis in starved cells as suggested by Doetsch [86] in his "Retromutagenesis model." According to this model, mutated proteins could allow stressed, non-growing cells to switch from a non-growth to a growth state where DNA replication is resumed. Then TLS polymerases could perform error-prone DNA synthesis in front of unrepaired lesions, fixing the mutation.

CONCLUDING REMARKS

More than twenty years of research have documented stress-induced mutagenesis in both Gram$^-$ and Gram$^+$ bacterial species and in yeast [8–10; 87,88]; however, it remains contentious if beneficial mutations (directed mutations) might arise driven by transcription [78–80; 84]. The phenomenon is a collection of mechanisms with similarities and differences among different bacterial species. Notwithstanding the huge amount of data in the field, an important question has yet to be answered: is stress-induced mutagenesis a relevant and widespread phenomenon in bacterial species in natural environments? To the best of our knowledge the only attempt to learn about stress-induced mutagenesis in *E. coli* natural isolates [75] have been abolished by further data, showing the limits of the experimental setup [76]. More efforts are needed to answer the aforementioned question. In planning future studies it should be considered that laboratory strains could have diverged significantly from the natural species. Moreover, conditions like biofilm formation / platonic life could affect the response of bacteria to environmental stresses with possible consequences on the formation and selection of stress-induced mutants. In our opinion, it would be worth concentrating efforts to envisage new experimental approaches instead of further investigating the molecular aspects of artificial systems such as the widely explored *lac* system.

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СРЕДОВОЙ СТРЕСС И МУТАГЕНЕЗ У КИШЕЧНЫХ И НЕКИШЕЧНЫХ БАКТЕРИЙ

Бабудри Н., Лансиони Х., Ачилли А.

❀ **SUMMARY: Трудно переоценить значение мутаций для эволюции. В течение долгих лет считалось, что мутагенез происходит только в делящихся клетках. К настоящему моменту стало ясно, что мутации происходят также в неделящихся или медленно делящихся клетках микроорганизмов. Естественные популяции большую часть времени находятся в стрессирующих условиях, когда скорость роста существенно снижена. Поэтому мутационный процесс, независимый от деления клеток (мутагенез, индуцированный стрессом, МИС), может играть существенную роль в эволюции. В данной статье мы рассматриваем современное состояние этой проблемы на моделях кишечных бактерий. Мы обсуждаем различные экспериментальные системы, а также механизмы и модели, которые согласуются с огромным объемом данных, полученных за более чем двадцать лет исследований.**

❀ **КЛЮЧЕВЫЕ СЛОВА:** мутагенез; индуцированный стрессом; некишечные бактерии; мутагенез, ассоциированный с транскрипцией.

❀ Информация об авторах

Бабудри Нора — доцент. Кафедра генетики и микробиологии, Университет Павии. Виа Феррата 1, 27100, Павия, Италия. E-mail: babudri@unipg.it.

Лансиони Ховираг — степень в области биологических наук. Кафедра генетики и микробиологии, Университет Павии. Виа Феррата 1, 27100, Павия, Италия. E-mail: lancioni@kataweb.com.

Ачилли Алессандро — научный сотрудник. Кафедра генетики и микробиологии, Университет Павии. Виа Феррата 1, 27100, Павия, Италия. E-mail: alessandro. E-mail: achilli@unipg.it.

Babudri Nora — Associate Professor. Dipartimento di Biologia Cellulare e Ambientale, Universitá di Perugia. Via Elce di Sotto, 06100, Perugia,Italy. E-mail: babudri@unipg.it.

Lancioni Hovirag — PhD. Dipartimento di Biologia Cellulare e Ambientale, Universitá di Perugia. Via Elce di Sotto, 06100, Perugia, Italy. E-mail: lancioni@kataweb.com.

Achilli Alessandro — Researcher. Dipartimento di Biologia Cellulare e Ambientale, Universitá di Perugia. Via Elce di Sotto, 06100, Perugia, Italy. E-mail: alessandro.achilli@unipg.it.