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Causes and Consequences of DNA Repair Activity Modulation During Stationary Phase in *Escherichia coli*

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ABSTRACT *Escherichia coli* responds to nutrient exhaustion by entering a state commonly referred to as the stationary phase. Cells entering the stationary phase redirect metabolic circuits to scavenge any available nutrients and become resistant to different stresses. However, many DNA repair pathways are downregulated in stationary-phase cells, which results in increased mutation rates. DNA repair activity generally depends on consumption of energy and often requires *de novo* proteins synthesis. Consequently, unless stringently regulated during stationary phase, DNA repair activities may lead to an irreversible depletion of energy sources and, therefore to cell death. Most stationary phase morphological and physiological modifications are regulated by an alternative RNA polymerase sigma factor RpoS. However, nutrient availability, and the frequency and nature of stresses, are different in distinct environmental niches, which impose conflicting choices that result in selection of the loss or of the modification of RpoS function. Consequently, DNA repair activity, which is partially controlled by RpoS, is differently modulated in different environments. This results in the variable mutation rates among different *E. coli* ecotypes. Hence, the polymorphism of mutation rates in natural *E. coli* populations can be viewed as a byproduct of the selection for improved fitness.

KEYWORDS environment, stress, starvation, mutation rates, RpoS

INTRODUCTION

Bacteria usually live in environments in which the abundance of nutrients is low. In addition, available nutrients are rapidly removed by bacterial populations and/or by other competing organisms. Therefore, starvation is one of the most common stressful conditions for bacteria (Kjelleberg, 1993). Bacteria possess elaborate mechanisms to cope with such stress and reduce its negative impact on survival. Some bacterial species, like *Bacillus subtilis*, can trigger a developmental program that results in the formation of starvation and stress-resistant spores. Non-differentiating bacteria, like *Escherichia coli*, respond to nutrient exhaustion by entering a state of insignificant growth or

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apparent dormancy, commonly referred to as the stationary phase (Huisman *et al.*, 1996; Nystrom, 2004). Stationary-phase cells, can survive days or even months of nutrient deprivation.

The response of *E. coli* to nutrient exhaustion is a reversible and gradual change that is fine-tuned, depending on the quantity and quality of available nutrients (Peterson *et al.*, 2005). When a particular nutrient becomes limiting, cells increase the production of proteins that scavenge the limiting nutrient or other substances that belong to the same class of nutrients. When remaining nutrients are depleted, the cells starve and enter stationary phase. Cells entering stationary phase undergo various morphological and physiological changes (Huisman *et al.*, 1996; Nystrom, 2004). They become spherical, which may be beneficial during starvation conditions because the surface/volume ratio of the cell is decreased. Cells are smaller than logarithmic-phase cells due to the degradation of endogenous material that is reused for stationary phase survival. The cell membrane becomes less fluid, less permeable, and favors adhesion and aggregation. The cytoplasm is condensed, while the volume of the periplasm increases. Cells synthesize storage substances, like glycogen and polyphosphate, and protective substances such as trehalose. In stationary phase cells stable RNA and protein turnover is stimulated. The transcription apparatus is modified by the reduction of the number of RNA polymerase holoenzymes containing sigma factor RpoD (σ^{70}). At the same time, the number of the RNA polymerase holoenzymes containing sigma factor RpoS (σ^{S}, σ^{38}) increases. RpoD controls transcription of housekeeping genes, whereas RpoS controls transcription of stationary-phase-specific genes. The translation machinery is also modulated. A small stationary-phase protein, Ribosome Modulation Factor (RMF), which is involved in the dimerization of 70S ribosome monomers giving rise to translationally incompetent 100S ribosomes, is produced. The number of ribosomes and rRNA gene expression decreases resulting in a reduction in the rate of global protein synthesis. Metabolic circuits are redirected to scavenge any potential nutrient from the medium or within the cell.

Cells that have entered stationary phase due to the exhaustion of different nutrients trigger the pathway to stationary phase in a different fashion (Peterson *et al.*, 2005). However, a subset of proteins synthesized during the transition to stationary phase is induced regardless of the class of nutrients for which cells are starved (Groat *et al.*, 1986). Matin and colleagues (1989) have proposed that these proteins are involved in stress protection. Stationary phase bacteria are very resistant to many stresses, such as heat shock, oxidative shock, osmotic shock, extremely low pH, UV, alkylating agents. An increased resistance is mostly the result of the activity of systems involved in protection of DNA, proteins, and membranes. The specificity of multiple-stress resistance of stationary phase cells is that the protection is preventive, *i.e.*, it is not induced by an external stress.

However, the proper maintenance of genetic information seems not to be assured during stationary phase, as an increase in mutation rates and genomic rearrangements have been observed in starved stationary-phase cells (Bjedov *et al.*, 2003; Foster, 2005; Hersh *et al.*, 2004). Cells possess a multitude of molecular mechanisms, which either eliminate damaged DNA from the genome, or which alleviate potentially deleterious interference of DNA lesions with normal DNA metabolism (Friedberg *et al.*, 2006). Consequently, in growing cells, most DNA lesions are efficiently repaired and mutation rates are kept at a minimum. In stationary-phase cells, cellular DNA repair capacity could be diminished due to the relocation of the starving cell's limited resources from repair to protection mechanisms because DNA repair activity generally depends on consumption of energy and often requires *de novo* synthesis of numerous proteins. In this review, the current knowledge on the state of genomic DNA and on the regulation of different DNA repair mechanisms in stationary phase *E. coli* cells is presented. Ecological and evolutionary implications of DNA repair activity modulation in stationary phase cells are also discussed.

GENOME CONTENT AND GENOME ORGANIZATION IN STATIONARY-PHASE CELLS

During late exponential and early stationary phase, the initiation of *E. coli* chromosome replication is inhibited by an, as yet uncharacterized, extracellular factor (Withers *et al.*, 1998). The inhibition of the initiation of chromosomal replication seems to be mediated by direct interaction with the replication machinery. This inhibition does not block ongoing replication to proceed to termination before complete exhaustion of nutrients. Otherwise, replication forks

may stall upon entry into stationary phase, which is a potentially deleterious event.

When *E. coli* cells are grown in a rich complex medium, the majority of stationary-phase cells contain 2, 4, or 8 chromosome equivalents (Akerlund *et al.*, 1995). The average DNA content decreases during the first days in stationary phase, but even after 6 days, a majority of cells contain more than one chromosome equivalent. Cells grown in a glucose minimal medium, on the other hand, end up with mostly 1 or 2 chromosome equivalents in stationary phase. This observation indicates that the termination of chromosomal replication does not lead necessarily to cell division. The presence of multiple chromosomes in stationary-phase cells may allow recombinational repair to occur at higher frequency than generally assumed for cells in stationary phase. Independently of the number of chromosome equivalents, a majority of stationary phase cells contain only one nucleoid, suggesting that chromosomal DNA is highly compacted.

The genomic DNA of *E. coli* is associated with about 10 different molecular species of DNA-binding structural protein, which together form the nucleoid (Ali Azam *et al.*, 1999). In exponential phase, Fis (factor for inversion stimulation), HU (heat-unstable nucleoid protein) and Hfq (host factor for phage Qβ; RNA chaperon) are the major structural components of the *E. coli* nucleoid. In early stationary phase, these proteins are replaced by Dps, IHF (integration host factor), and CbpA (curved DNA-binding protein A of unknown function). The level of Dps, IHF, and CbpA proteins also increases in cells that are slowly growing in poor medium. In late stationary-phase cells, Dps becomes the most abundant protein (180,000 molecules/cell) and approaches almost half of the level of total nucleoid-bound proteins. Dps proteins are distributed uniformly within the entire nucleoid (Azam *et al.*, 2000). Dps organizes the chromosome into one, tightly packed, highly ordered, crystalline structure that is resistant to oxidative, thermal, acid and base stresses; UV and gamma irradiation; and iron and copper toxicity (Frenkiel-Krispin *et al.*, 2004; Kim *et al.*, 2004; Nair *et al.*, 2004). The protective role of Dps is performed through a combination of chromosome compaction, metal chelation, ferroxidase activity, and regulation of gene expression. The up-regulation of *dps* gene transciption in stationary phase cells is controlled by RpoS RNA polymerase sigma factor.

TRANSCRIPTION REGULATION DURING STATIONARY PHASE

RpoS is one of seven *E. coli* RNA polymerase sigma factors, which compete for association with the core polymerase subunit (Eisenstark *et al.*, 1996; Hengge-Aronis, 2002). The outcome of the competition is influenced by the varying number of each sigma factor and by different molecules that can affect the binding of sigma factors to the RNA polymerase. Each sigma factor coordinates the transcription of a set of genes, thus allowing fine control of adaptation to different physiological condition. The production of RpoS is regulated at every step of gene expression: transcription, translation, protein stability, and activity. Transcription of the *rpoS* gene is controlled by the cAMP receptor protein and through the signaling of ppGpp and polyphosphate. The *rpoS* mRNA is translated at low levels due the hairpin structure that occludes the Shine-Dalgarno sequence. The stability of this *rpoS* mRNA secondary structure is modulated by a cascade of interacting factors, including Hfq, HU, H-NS (histone-like nucleoid structuring protein), LeuO (transcription regulator), and by small noncoding RNAs, *dsrA* RNA, *rprA* RNA and *oxyS* RNA. In growing cells, RpoS levels are maintained at a low level due to degradation by the ClpXP protease in a reaction that is promoted by RssB (proteolytic targeting factor) and inhibited by the chaperone DnaK. Various stress conditions differentially affect the mechanisms of control of RpoS concentration (Hengge-Aronis, 2002; Peterson *et al.*, 2005). Thus, a reduced growth rate results in increased *rpoS* transcription whereas high cell density, high osmolarity, low temperature, phosphorus starvation, and low pH stimulate the translation of already present *rpoS* mRNA. Low pH, carbon source starvation and high temperature modulate RpoS proteolysis.

When present at high concentration, RpoS outcompetes the primary sigma factor, RpoD, and regulates transcription of hundreds of genes with unrelated physiological functions (Patten *et al.*, 2004; Weber *et al.*, 2005). Several factors have been shown to determine the outcome of this competition. Lrp (leucine-responsive regulatory protein) affect the selectivity of these two sigma factors for many promoters. Rsd (regulator of Sigma D), an anti-RpoD factor, controls the level of functional RpoD holoenzyme (Jishage *et al.*, 1998). Expression of the *rsd* gene correlates inversely

with growth rate. The intracellular concentrations of glutamate, and polyphosphate, as well as, decreased DNA superhelicity have also been shown to enhance the activity of RpoS holoenzyme and to repress that of RpoD in stationary phase *E. coli* cells. Because the RpoS regulon is not induced only in stationary phase, but it responds to many different stress conditions, it is considered a general stress response (Hengge-Aronis, 2000). The induction of the RpoS regulon results in a cessation of growth and general stress protection. Hence, the response of the cell to one stress promotes protection to the encountered stress and, additionally, to a variety of other stresses.

OXIDATIVE DAMAGE REPAIR DURING STATIONARY PHASE

Reactive oxygen species are deleterious to cells because they can damage proteins, DNA and membranes. The RpoS regulon protects stationary phase cells from reactive oxygen species, as *E. coli rpoS* mutants are rapidly killed by exogenous oxidative stress (Eisenstark *et al.*, 1996). Transcriptome analysis of *E. coli rpoS* strain colonies show higher expression of the *oxyR* and *soxR* genes (coding for transcriptional regulators involved in the response to hydrogen peroxide and superoxide, respectively (Storz *et al.*, 2000) compared with wild-type colonies, indicating that endogenous oxidative stress is a major problem for stationary-phase *rpoS* cells (Saint-Ruf *et al.*, 2004). The induction of the *oxyR* and *soxR* stress responses in a *rpoS* mutant allows it to overcome the deficiency in RpoS-mediated protection against oxidants. This protection is assured by upregulation of the following genes: *gorA* coding for glutathione oxidoreductase, *sodC* coding for periplasmic superoxide dismutase that detoxify superoxide anions, and *katE* and *katG* coding for catalases that detoxify hydrogen peroxide. Genomic DNA is protected from oxidative damage by the aforementioned Dps. Besides being regulated by RpoS, *katG*, *dps* and *gor* genes are also regulated by OxyR (Storz *et al.*, 2000). In stationary phase, RpoS upregulates expression of the *xthA* gene, which codes for Exonuclease III, which has 5′ AP (apurinic and apyrimidinic sites) endonuclease activity (Sak *et al.*, 1989). The fact that *xthA* mutants are hypersensitive to hydrogen peroxide clearly indicate that Exonuclease III participates in repair of oxidative damage. In addition to induction of the above-mentioned RpoS-regulated genes that contribute to protection against oxidative agents, the transcription of *ahpCF* and *sodA,* coding respectively for alkyl hydroperoxide reductase and manganese-containing superoxide dismutase, is also stimulated in stationary phase (Michan *et al.*, 1999).

Despite the induction of multiple protective functions against reactive oxygen species in stationary phase cells, genomic DNA is not fully protected. For example, the artificial overproduction of Fpg and MutY glycosylases reduces the rate of mutagenesis in stationary-phase cells (Bridges *et al.*, 1996). Formamidopyrimidine DNA glycosylase (Fpg) and MutY DNA glycosylase work together to protect cells from the mutagenic effects of the common oxidative damage 7,8-dihydro-8-oxoguanine (8-oxoG). Fpg removes 8-oxoG from 8-oxoG-C pairs, giving the DNA polymerase a chance to put in G. If 8-oxoG is not removed before DNA replication occurs, it can mispair with A. MutY removes A in 8-oxoG-A mispairs. Failure of this process results in a G:C→T:A transversion. In stationary-phase cells, G:C→T:A transversions indeed arose at high frequency (Mackay *et al.*, 1994). It has been calculated that the rate of production of 8-oxoG in the DNA of starved cells is threefold greater than in the DNA of growing cells (Bridges *et al.*, 1996). It is therefore surprising that *fpg* and *mutY* genes are downregulated in stationary-phase cells (Gifford *et al.*, 2000).

In addition, *nth* and *nei* genes, coding respectively for endonuclease III and endonuclease VIII are also downregulated in stationary-phase cells (Gifford *et al.*, 2000). These two enzymes have overlapping substrate specificities and recognize and remove a wide range of oxidized pyrimidines. Some of these oxidized pyrimidines, such as thymine glycol, act as blocks to DNA polymerase and are lethal to cells; oxidized cytosines such as uracil glycol, 5-hydroxyuracil, and 5-hydroxycytosine pair with A and are premutagenic, leading to G:C→A:T transitions. The decrease in transcripts seen for *fpg*, *mutY*, *nth*, and *nei* is not the result of repression by the RpoS regulated gene.

The transcription of *fpg*, *mutY*, *nth*, and *nei* increases when cells are shifted from anaerobic to aerobic growth conditions (Gifford *et al.*, 2000). Such a shift results in an important generation of superoxide generated by fumarate reductase, an anaerobic terminal oxidase, which is abundant in cells under anaerobic conditions (Imlay, 1995). The induction of *fpg*, *mutY*, *nth*, and

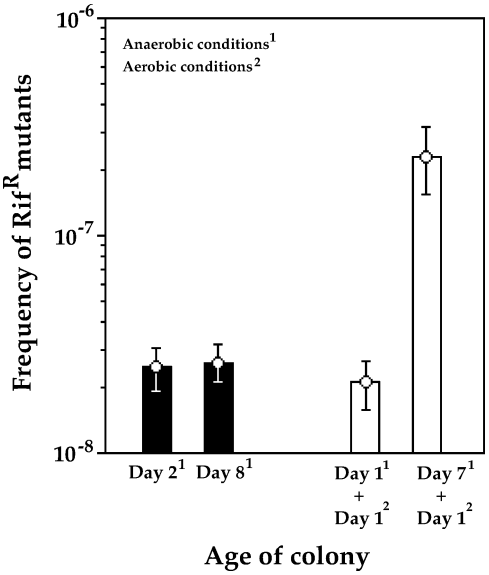

Figure 1 **Mutation frequency increases upon shift from anaerobic to aerobic growth conditions in old, but not in young *Escherichia coli* C4750 colonies. Mutation frequency was estimated by measuring frequency of rifampicin resistant mutants in bacterial colonies at indicated time points. Every spot represents the mean value (+/− SE) from three independent experiments. Experimental protocol is described in (Bjedov *et al.*, 2003). These data show that the capacity of stationary phase cells to protect DNA upon shift from anaerobic to aerobic conditions diminishes with time. C4750 is a natural isolate described previously (Bjedov *et al.*, 2003).**

nei may be sufficient to protect growing cells, but not stationary-phase cells, upon such a shift. For example, we observed that, upon shift to aerobic conditions, mutation frequency increases significantly in a stationary phase colony previously grown and incubated under anaerobic conditions for seven days (Figure 1). Such an increase in mutation frequency is not observed when the *E. coli* C4750 colony was incubated under anaerobic conditions for only one day before shift to aerobic conditions. Therefore, the capacity of stationary-phase cells to protect DNA upon shift from anaerobic to aerobic conditions diminishes with time. We expect that usually, bacteria are submitted to such shift in colonies, as colonies are nearly completely anaerobic during the first days of growth because of intense metabolic activity of the bacteria on the surface of the colony (Peters *et al.*, 1987). When nutrients are exhausted, metabolic activity slows, and oxygen penetrates the colony, which results in an increase in mutation frequency. This is borne out by the observation of that mutant frequency increased in the colonies of C4750 strain kept "aerobic" for the entire eight days (Bjedov *et al.*, 2003) to similar extent as upon shift from anaerobic to aerobic conditions (Figure 1).

ALKYLATION DAMAGE REPAIR DURING STATIONARY PHASE

Alkylating agents are extremely cytotoxic and mutagenic DNA damaging agents that are produced endogenously in cells and present in the environment. *E. coli* posses an inducible alkylation-specific DNA repair response called the adaptive response, which encompass *ada*, *alkA*, *alkB*, and *aidB* genes (Friedberg *et al.*, 2006; Sedgwick *et al.*, 2007). Ada protein, an O6-methylguanine-DNA methyltransferase, removes methyl groups from methylated bases thus eliminating miscoding alkylating DNA lesions. Ada protein also regulates the adaptive response. The methylated Ada protein interacts with the promoters of the genes from the adaptive response regulon and activates their transcription. AlkA is a DNA glycosylase that excises a variety of damaged bases including cytotoxic 3-methyladenine and 3-methylguanine. AlkB is a DNA dioxygenase that removes the methyl groups from the alkylated bases 1-methyladenine and 3-methylcytosine by oxidative demethylation. The exact role of AidB is not established, but it was shown that it provides some protection against methylating agents.

RpoS intervenes in the regulation of expression of genes from the Ada regulon (Landini *et al.*, 1999). Methylated Ada protein activates RpoS-dependent induction of *ada* and *aidB* genes and RpoS-dependent repression of the *alkA* gene (Taverna *et al.*, 1996; Volkert *et al.*, 2001). The inactivation of *ada* and *ogt* (coding for constitutive O6-methylguanine-DNA methyltransferase) genes does not increase mutation rates in growing cells, but it does increase mutation rates in stationary phase cells (Mackay *et al.*, 1994; Taverna *et al.*, 1996). This observation further confirms the presence of alkylating DNA damage in stationary-phase cells. It was proposed that spontaneous endogenous generation of alkylating agents increases during stationary phase due to nitrosation of amino acids (Taverna *et al.*, 1996).

MISMATCH REPAIR DURING STATIONARY PHASE

The methyl-directed mismatch repair (MMR) system maintains the integrity of the genome by controlling

the fidelity of DNA replication and by preventing recombination between nonidentical DNA sequences (Friedberg *et al.*, 2006). Three genes are specifically dedicated to MMR in *E. coli*: *mutS*, *mutL* and *mutH*. The MutS protein homodimer recognizes and binds to unpaired and mispaired nucleotides in duplex DNA. MutL homodimer associates with the MutS-mismatch complex and activates MutH, which incises the newly synthesized strand at hemimethylated 5′-GATC-3′ sites. GATC sites are hemimethylated because methylation of adenine by the Dam methylase lags behind replication by several minutes. Hence, MutH directs excision and resynthesis to the newly synthesized strand performed by other proteins. The inactivation of MMR genes gives strong mutator phenotypes. MMR mutants have a 10^2 to 10^3-fold increased rate of transition (both G:C→A:T and A:T→G:C) and frameshift (insertion/deletion of a few nucleotides) mutations, compared with MMR proficient bacteria. MMR antirecombinogenic activity maintains integrity of chromosomes by preventing recombination between repeated DNA sequences and by aborting recombination with foreign DNA acquired through horizontal gene transfer (Matic *et al.*, 1996).

In stationary-phase cells, the transcription of *mutS* and *mutH* genes and the concentration of MutS and MutH proteins decreases to very low levels *via* an RpoS and Hfq dependent mechanism, compared to growing cells (Feng *et al.*, 1996; Harris *et al.*, 1997; Tsui *et al.*, 1997). Consequently, the activity of the MMR system is reduced during stationary-phase. Transfection experiments of phage DNA containing heteroduplexes show that mismatch repair capacity is indeed decreased in stationary-phase cells and that this reduced repair capacity can be reversed by overexpression of MutS but not MutL protein (Saint-Ruf *et al.*, 2006). Overproduction of the MutS protein in wild-type cells significantly decreased stationary-phase mutagenesis (Bjedov *et al.*, 2003) but not mutagenesis in growing cells. Similarly, overexpression of the MutS repair protein significantly decreased the rate of G:C→T:A transversion mutation in stationary-phase wild-type, *mutY* and *mutM* strains (Zhao *et al.*, 2000).

Under carbon source starvation mismatch repair can be also be inefficient because MutL protein becomes limiting (Rosenberg *et al.*, 2003). It is known that MutL protein can be titrated by errors made by an error-prone mutant DNA polymerase III (Schaaper *et al.*, 1989), by overproduction of Vsr protein, which interacts with MutL (Doiron *et al.*, 1996), by treating cells with base analogs, like 2-aminopurine (Matic *et al.*, 2003), and by overproduction of error-prone DNA Pol IV (Wagner *et al.*, 2000). As PolIV is responsible for increase of mutagenesis during carbon source starvation (Harris *et al.*, 1997; McKenzie *et al.*, 2001), it is conceivable that MutL protein is titrated by the by errors made by Pol IV in starved cells.

While MMR is more efficient during exponential growth, specialized very-short-patch (VSP) repair is more efficient during stationary phase. VSP repair corrects T:G mismatches created by 5-methylcytosine deamination to thymine (Macintyre *et al.*, 1999). The repair process is initiated by a single-stranded endonuclease, Vsr, which cleaves 5′ of the mismatched T. Removal of several bases 3′of the nick and their resynthesis by DNA polymerase I complete the repair process. Vsr is undetectable during the exponential growth phase, appearing only as the cells enter stationary phase. Vsr disappears very rapidly when stationary-phase cells are diluted into fresh medium, suggesting that the protein is actively degraded as cells prepare to re-enter growth phase.

DNA REPAIR AND TRANSCRIPTION ERRORS DURING STATIONARY PHASE

Unrepaired DNA damages poses yet another problem for stationary phase cells because they can be mutagenic for ongoing transcription. For example, it was shown that RNA polymerase slips over 8-oxoG generating deletions in the transcript, and that it inserts adenine or cytosine opposite to 8-oxoG in the DNA of nondividing *E. coli* cells (Bregeon *et al.*, 2003). Such transcriptional mutagenesis can considerably increase production of mutant proteins in stationary phase cells because (i) the amount of 8-oxoG in the DNA of starved cells was shown to be greater than in the DNA of growing cells (Bridges *et al.*, 1996), and (ii) DNA glycosylases Fpg and MutY, which work together to eliminate 8-oxoG from DNA are downregulated during stationary phase (Gifford *et al.*, 2000).

RNA polymerase also efficiently inserts adenine opposite to uracil, resulting in G to A base substitutions in nondividing *E. coli* cells producing a pure population of mutant proteins (Viswanathan *et al.*, 1999). Therefore, uracil must be removed from DNA before its encounter with the RNA polymerase. Uracil in DNA can arise

as a result of either misincorporation of dUTP by DNA polymerase, or by deamination of DNA cytosine (Friedberg *et al.*, 2006). In growing cells, Uracil-DNA glycosylase (Ung) encoded by the *ung* gene removes uracil from DNA. However, the *ung* gene is repressed in stationary phase (De Wulf *et al.*, 2002; Varshney *et al.*, 1988). *ung* gene transcription is controlled by the two-component The Cpx system comprising the CpxA sensor kinase and the CpxR response regulator. The Cpx system coordinates a response to a variety of stresses including membrane-protein damage, starvation, and high osmolarity. At the onset of the stationary growth phase, the phosphorylated CpxR activates *cpxRA* expression in conjunction with RpoS resulting in the increased expression of *cpxRA* (De Wulf *et al.*, 1999), which results in repression of the *ung* gene (Ogasawara *et al.*, 2004). Overexpression of CpxR results in increased production of G:C→A:T transition mutations, which are a signature of *ung* deficiency. The effect of *ung* gene downregulation in stationary phase is not attenuated by the RpoS-dependent induction of *mug* (mismatched uracil glycosylase) gene. Although Mug glycosylase can excise uracil and 3,N4-ethenocytosines from DNA (Mokkapati *et al.*, 2001), it seems that uracil is not its primary substrate in stationary phase cells.

Transcriptional mutagenesis contributes to the production of mutant proteins together with translational frameshifting, missense errors, and stop codon readthrough that increase in stationary phase *E. coli* cells (Nystrom, 2004). This may be one of the explanations for why DnaK, GroEL, and HtpG heat shock proteins, as well as RpoH (σ^{32}), a heat shock specific sigma factor, are induced in stationary phase *E. coli* cells (Jenkins *et al.*, 1991). The induction of the *rpoH* gene is necessary for survival of starving cells, as a *rpoH* knockout strain was shown to be sensitive to carbon starvation. Therefore, unrepaired DNA damage may contribute to the reduction of the capacity of cells to survive starvation during stationary phase by contributing to transcriptional mutagenesis.

DNA REPAIR DOWNREGULATION DURING STATIONARY PHASE

The most frequently invoked reason for downregulation of the above-mentioned DNA repair functions is based on the assumption that there is no DNA replication during stationary phase. It is true that the toxicity or mutagenicity of many DNA lesions is revealed only during DNA replication. A good example is down-regulation of the *alkA* gene in stationary phase, which codes for AlkA protein that removes replication-blocking 3-methyladenine and 3-methylguanine (Landini *et al.*, 1999). Therefore, in the absence of replication, these DNA lesions are expected not to be toxic. In addition, *E. coli* posses another 3-methyladenine-DNA glycosylase encoded by the *tag* gene, which can efficiently repair these lesions in the absence of AlkA. However, the proposed hypothesis may not explain all, because the same logic should be applied to the Ada protein, which removes O6-methylguanine that is mutagenic only during DNA replication. However, the Ada protein is overproduced during stationary phase although this lesion can efficiently be repaired by another O6-methylguanine-DNA methyltransferase, the *ogt* gene product. Inactivation of both *ada* and *ogt* genes increases mutation rates in nongrowing stationary phase cells, but not in growing cells (Mackay *et al.*, 1994; Taverna *et al.*, 1996). An additional indication that the proposed hypothesis is not fully satisfactory is provided by observations that an overproduction of some of the downregulated DNA repair functions, e.g., MutS or MutY, does reduce stationary phase mutagenesis (Bjedov *et al.*, 2003; Bridges *et al.*, 1996; Zhao *et al.*, 2000). Finally, there are direct indications that DNA is synthesized in stationary phase cells.

The expression of *dnaN* and *recF* genes, increases significantly in stationary-phase cells (Villarroya *et al.*, 1998). The *dnaN* gene encodes a β sliding clamp responsible for tethering the DNA polymerase to DNA and endowing it with high processivity, while *recF* codes for a DNA-binding protein involved in recombination, repair and in the resumption of DNA replication at disrupted replication forks. The induction of these two genes is dependent on RpoS, and independent of the *dnaA* gene promotor from which *dnaN* and *recF* are expressed in exponentially growing cells. The induction of the *dnaN* gene indicates that the DNA polymerase III holoenzyme and/or the other four *E. coli* polymerases are involved in the maintenance of DNA integrity under stationary phase. It was indeed reported that *E. coli* PolII, PolIV, and PolIV, involved in repair and tolerance of DNA lesions, are required for long-term survival in stationary phase (Yeiser *et al.*, 2002). A β sliding clamp is assembled onto DNA by the

ATP-dependent clamp-loading activity of the gamma complex. Addition of a large excess of β sliding clamp subunits to the holoenzyme circumvents the need for ATP in forming a processive polymerase. Since ATP levels decrease as cells go from the exponential to the stationary phase of growth (Walker *et al.*, 2004), it is possible that the induction of the *dnaN* gene would be required to guarantee activity of DNA polymerases in starved cells in spite of low ATP levels.

How important DNA synthesis can be in nongrowing *E. coli* cells is illustrated by the study of Tang and colleagues (1979). They measured the amount of radioactive thymidine incorporated into, and lost from, the genome of *E. coli* cells incubated in buffer at 37°C for 24 hours. During this time there was no net change in cellular DNA content, but at least 20% of the genome was broken down and resynthesized. The synthesis was reduced by 90% in a *polA* mutant. As the *polA* gene codes for DNA polymerase I, which is involved in various DNA repair activities (Friedberg *et al.*, 2006), the observed DNA synthesis was assumed to be repair synthesis.

Induction of the *dinB* gene coding for DNA polymerase IV during stationary phase is another argument for the existence of DNA synthesis in stationary-phase cells (Layton *et al.*, 2003). This DNA polymerase is capable of bypassing DNA lesions, which block chain elongation by the replicative DNA polymerase (Jarosz *et al.*, 2006). Translesion synthesis is often performed with high fidelity, but PolIV exhibits reduced fidelity when it operates on undamaged DNA or on noncognate DNA lesions (Fuchs *et al.*, 2004). During growth phase, the expression of the *dinB* gene is under the control of the SOS regulon (Friedberg *et al.*, 2006). SOS is induced by DNA damage and blockage of DNA replication that increase the intracellular concentration of single-strand DNA. The persistent contact with single-strand DNA activates the co-protease activity of the RecA protein, which promotes the self-cleavage of the LexA protein (the SOS repressor) thus inducing the SOS response. The *E. coli* SOS system is composed of at least 40 genes, many of which code for DNA repair functions, *e.g.*, nucleotide excision repair, translesion-synthesis and homologous recombination (Courcelle *et al.*, 2001). The SOS system was shown to be induced in aging *E. coli* colonies, and this induction results in an increase of mutant frequency (Taddei *et al.*, 1995).

Under carbon source starvation and stationary phase, the expression of the *E. coli dinB* gene is elevated, resulting in the increased generation of stationary phase mutations (Layton *et al.*, 2003; McKenzie *et al.*, 2001). The induction of *dinB* gene transcription during stationary phase is controlled by RpoS and, only partially, by LexA (Layton *et al.*, 2003). Such noncanonical regulation of DNA polymerase genes may help the cells to survive certain damages frequently inflicted on the genome during prolonged stationary phase, like cytotoxic alkylating DNA lesions (Bjedov *et al*., 2007). Unlike the replicative polymerase PolIII, PolIV and PolV have the potential to operate efficiently at low dNTP concentrations, a condition encountered during stationary phase (Godoy *et al.*, 2006). This may explain why PolIV and PolV are required for long-term survival in stationary phase.

Taken together, the above-mentioned observations indicate that there is DNA synthesis in stationary phase cells. Some is probably linked with DNA repair activity, while some could be genuine chromosome replication in the cells that scavenge nutrients released by dead cells. Therefore, stationary phase-specific downregulation of DNA repair genes results in the increased generation of mutations because there is DNA synthesis in stationary-phase cells. However, the longer-term fitness cost incurred by the generated deleterious mutations is probably much less important than the immediate cost of DNA repair activity under starvation conditions. DNA repair activity depends on the synthesis of enzymes, whose activities are ATP dependent. While such a requirement can be efficiently met as long as nutrients are abundant, it is difficult to satisfy it in cells, in which the main source of energy is provided by the degradation of endogenous components. Consequently, unless stringently regulated during stationary phase, DNA repair activities may lead to an irreversible depletion of energy sources and, therefore, to the cell death.

However, there is another, nonexclusive, hypothesis as to why DNA repair mechanisms are downregulated during starvation. The activity of some DNA repair enzymes may be deleterious in stationary phase because DNA repair intermediates can be toxic for the cell. For example, DNA glycosylases generate abasic sites and strand breaks, which require intervention of many other DNA repair enzymes in order to restore original genetic integrity. Such DNA lesions may not be efficiently repaired in stationary-phase cells. Similarly, MMR activity can damage DNA carrying lesions when all GATC sequences are not methylated. For example, *E.*

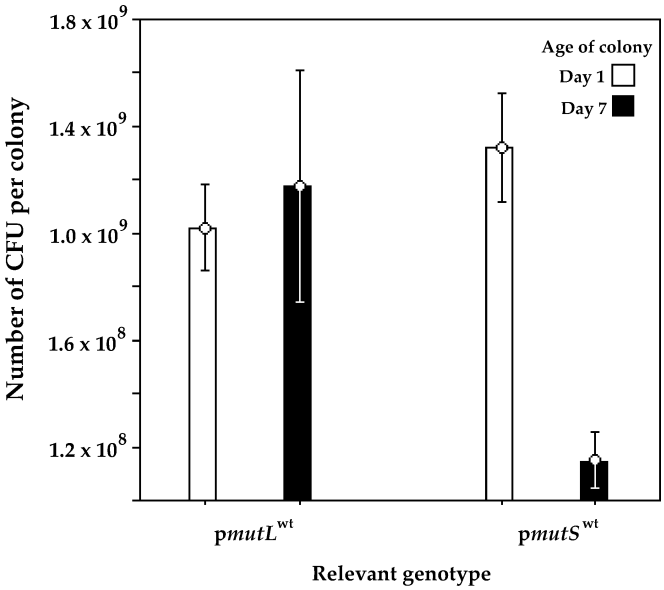

Figure 2 **Overexpression of MutS, and not of MutL, decreases the number of colony forming units in seven-day old *Escherichia coli* MG1655 colonies. Every bar represents the mean value (+/– SE) from three independent experiments. Experimental protocol is described in (Bjedov *et al.*, 2003). These results, together with the fact that the *mutS* gene, and not the *mutL* gene, is downregulated during stationary phase suggest that the *mutS* gene is repressed during stationary phase not only because MutS protein is "useless," but also because it may be "dangerous" under prolonged starvation conditions. MG1655 is the sequenced "wild-type" *E. coli* K12 laboratory strain (Blattner *et al.*, 1997).**

coli dam cells have an increased sensitivity to hydrogen peroxide that can be suppressed by inactivating MMR (Wyrzykowski *et al.*, 2003). Also, an active MMR system renders *S. enterica dam* mutants sensitive to bile salts (Prieto *et al.*, 2004). We observed that the expression of the *dam* gene is significantly reduced in *E. coli* cells after 7 days in stationary phase relative to early stationary phase (data not shown). Therefore, DNA synthesized under those conditions might be nonmethylated. We also observed that overproduction of MutS protein, but not that of MutL protein, is deleterious in stationary phase (Figure 2). This indicates that some DNA repair functions are repressed during stationary phase not only because they are "useless," but also because they are "dangerous" under starvation conditions.

ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS OF STATIONARY PHASE-ASSOCIATED PHENOTYPES AMONG NATURAL ISOLATES

The availability of essential nutrients, including carbon, nitrogen, and phosphorus, differ in different ecological niches. In addition, the nature and the frequency of different stresses differ in different ecological niches. Therefore, it is not surprising that different *E. coli* ecotypes, which have different lifestyles, have different nutritional capabilities and respond differently to the same stresses. In many cases, such variability was shown to result from allelic variation of *rpoS* gene or from polymorphism of its regulatory elements (Ferenci, 2003). For example, strains with high intracellular levels of RpoS protein (i) metabolize fewer substrates and poorly compete for low concentrations of nutrients, and (ii) have increased stress resistance (King *et al.*, 2004). The reduction of the intracellular concentration of RpoS has exactly the opposite effects on nutritional competence and stress resistance. These phenotypes are, to a large degree, the consequence of a competition between different sigma factors within the cell, *e.g.*, the absence of RpoS allows a higher level of transcription of RpoD-dependent genes (Nystrom, 2004). Therefore, nutritional limitations and environmental stress conditions impose conflicting choices that result in selection of the loss or modification of RpoS function in different environmental niches in function of nutrient availability and of frequency and nature of stresses.

E. coli strains that have increased stress resistance due to high intracellular levels of the RpoS protein have also increased mutation rates (King *et al.*, 2004; Yang *et al.*, 2004). This can be explained by the RpoS-dependent down-regulation of DNA repair genes discussed in this review. As we observed that there is a positive correlation between intracellular concentrations of RpoS protein among *E. coli* natural isolates and spontaneous mutation frequency (unpublished observation), it can be concluded that different amounts of RpoS protein differently affect DNA repair activity. It was also observed that the level of stationary phase dependent MMR system down-regulation is different in different *E. coli* natural isolates (Li *et al.*, 2003). Hence, the variable rates of generation of genetic variability of natural isolates can be viewed as a byproduct of the selection for improved fitness (Tenaillon *et al.*, 2004). This can explain why the ecological niche, from which the strain was isolated, appears to be the major determinant of a strain's mutator phenotype. For example, host diet and bacterial lifestyle, *i.e.* commensal and pathogenic, was shown to correlate with the variability of stationary-phase mutation frequencies among *E. coli* natural isolates. As even a very modest modification

of mutation rates can significantly influence bacterial evolution (Denamur *et al.*, 2005; Komp Lindgren *et al.*, 2003), the above described modulation of mutations rates is expected to have an impact on bacterial adaptive evolution. Evolution is interplay between genetic variation and phenotypic selection. Because genetic variation is primarily produced by mutations, different mutation rates modulate the probability of generation of adaptive variants, *e.g.*, higher mutation rates generate more adaptive mutations (Denamur *et al.*, 2006).

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