DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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THE ROLE OF DNA MISMATCH REPAIR AND OXIDATIVE DNA DAMAGE DEFENSE SYSTEMS IN AVOIDANCE OF STATIONARY PHASE MUTATIONS IN *PSEUDOMONAS PUTIDA*

SIGNE SAUMAA

TARTU UNIVERSITY
PRESS

Department of Genetics, Institute of Molecular and Cell Biology, University of Tartu, Estonia

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Supervisor: Dr. Maia Kivisaar, PhD (University of Tartu)

Opponent: Professor Elżbieta Grzesiuk, PhD (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Poland)

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications referred to in the text by their Roman numerals:

I **Saumaa, S., Tover, A., Kasak, L. and Kivisaar, M. 2002.** Different spectra of stationary-phase mutations in early-arising versus late-arising mutants of *Pseudomonas putida*: involvement of the DNA repair enzyme MutY and the stationary-phase sigma factor RpoS. *J. Bacteriol* **184**: 6957–6965**.**

II **Saumaa, S., Tover, A., Tark, M., Tegova, R. and Kivisaar, M. 2007.** Oxidative DNA damage defense systems in avoidance of stationary-phase mutagenesis in *Pseudomonas putida*. *J. Bacteriol* **189**: 5504–5514**.**

III **Saumaa, S., Tarassova, K., Tark, M., Tover, A., Tegova, R. and Kivisaar, M. 2006.** Involvement of DNA mismatch repair in stationary-phase mutagenesis during prolonged starvation of *Pseudomonas putida*. *DNA Repair* **5**: 505–514.

My contribution to the journal articles referred to in current dissertation is following:

Ref. I performed starvation assay experiments, mutation spectra analysis
Ref. II perfomed starvation assay experiments, mutation spectra analysis, experiments with Dps including oxidative stress assay, participated in writing of paper
Ref. III perfomed starvation assay experiments, mutation spectra analysis, participated in writing of paper

ABBREVIATIONS

AP site	apurinic/apyrimidinic site; abasic site
BER	base excision repair
bp	base pairs
CF	cystic fibrosis
Dam	DNA adenine methylase
dNTP	2´-deoxyribonucleoside-5´-triphosphate
Dps	stationary phase specific DNA-binding protein
DSB	double-strand break
Fapy	formamidopyrimidine
Gh	guanidinohydantoin
GO	7,8-dihydro-8-oxoguanine, 8-oxoG
IR	ionizing radiation
kDa	kilodalton
Lac	lactose
MAC	mutagenesis in aging colonies
MMR	DNA mismatch repair
NER	nucleotide excision repair
NHEJ	nonhomologous end-joining
nt	nucleotide
Nudix	nucleoside diphosphate linked to another component, X
Phe	phenol
Pol	polymerase
Rif	rifampicin
ROS	reactive oxygen species
SSB	single strand binding protein
Sp	spiroiminodihydantoin
UV	ultraviolet

INTRODUCTION

Natural microbial populations are constantly exposed to variable environmental conditions. Bacteria live in such changing habitats mostly in growth-restricting conditions, mainly starving for nutrients. When bacterial population is unable to grow because of either shortage of required nutrients or impossibility to use the currently available nutrient sources, mutants, able to overcome the growth barrier arise by a process known as stationary phase mutation. The process of stationary phase mutation encompasses different mechanisms that are induced as a response to variety of stresses that collectively aide bacteria to react appropriately to various environmental signals by enhancing a mutation frequency and hence, lead to genetic variability that consequently allows some members of the population to accomplish phenotype beneficial for survival.

As bacteria in natural habitats face long periods of nutrient deprivation, most of the conducted stationary phase mutagenesis studies have tried to mimic the natural conditions and used nonlethal selective pressure of starvation either for a carbon source or for an amino acid. One of the characteristic features of stationary phase mutation found is the specific stationary phase mutation spectrum, distinct from actively growing bacteria. This has led to the suggestion that there must be different molecular mechanisms responsible for creating mutations in starving and growing bacteria. However, there appears to be no single pathway for generating stationary phase mutations. The mechanisms increasing mutation frequency in starving bacteria include the enhanced proportion of mutagenic DNA synthesis conferred by error-prone DNA polymerases in response to DNA damage and malfunctioning of DNA repair activity. Also, genetic variability can be generated by the movement of transposable elements. It has been shown that oxidative DNA damage is an important source of stationary phase mutations as well. Damaged base such as oxidation product of guanine, 7,8-dihydro-8-oxoguanine (8-oxoG), is susceptible to misreading during DNA replication and directs frequent insertion of adenine instead of cytosine thereby leading to G:C-to-T:A transversions (Shibutani *et al.*, 1991). The recently discovered 8-oxoG secondary oxidation products are shown to be even more mutagenic than 8-oxoG itself as tend to block DNA replication (David *et al.*, 2007). In order to alleviate the mutagenic potential of 8-oxoG, there is oxidized guanine (GO) repair system utilizing DNA glycosylases belonging to base excision repair pathway. Failure to repair 8-oxoG either from DNA or nucleotide pool leads to the increase in mutation frequencies in starving bacteria (Bridges, 1996; Bridges *et al.*, 1996). On the other side, lack of the activity of stationary phase specific DNA-binding protein Dps in *E. coli* cells that is known to protect DNA against oxidative damage, also increases mutation frequency, although only in the presence of exogenously added hydrogen peroxide (Martinez and Kolter, 1997). However,

the role of Dps in stationary phase mutagenesis under physiological conditions has remained unknown.

When bacteria are exposed to DNA-damaging agents, the expression of specialized DNA polymerases Pol II, Pol IV and Pol V is induced as a part of the SOS stress response regulon (Goodman, 2002). Specialized DNA polymerases are involved in the increase of mutation rate of stationary phase bacteria as these polymerases can replicate through various kinds of lesions and such mutagenic DNA synthesis elevates genetic diversity. For example, error-prone DNA polymerase Pol IV has been shown to enhance frameshift mutations whereas stationary phase mutagenesis pathway that requires DNA polymerase V generates base substitutions in *E. coli* (Bhamre *et al.*, 2001; McKenzie *et al.*, 2001). The major role in the mechanism of guanine oxidation mutagenesis is accomplished also by Pol V (Neeley *et al.*, 2007) although, whether it occurs also in starving bacteria is not known.

While DNA replication by specialized DNA polymerases can introduce genetic diversity, DNA repair is designed to avoid mutations from occurring. Thus, the efficiency of DNA repair is another factor that controls mutation frequency. Among different DNA repair pathways, aforementioned GO repair and DNA mismatch repair (MMR) that corrects mispairs and insertion/deletion mismatches, have been implicated in stationary phase mutagenesis. It has been argued that MMR might be malfunctioning in starving cells thus facilitating the occurrence of stationary phase mutations (Foster, 1999b; Harris *et al.*, 1999). Later studies have suggested that MMR deficiency might be caused by its saturation with an excess of DNA replication errors (Negishi *et al.*, 2002). Another major error-correcting pathway is nucleotide excision repair that eliminates wide variety of bulky DNA helix distorting damage. However, its role in stationary phase mutagenesis is not clear yet (Bridges, 1998; Hall, 1995). Overall, as all processes leading to stationary phase mutation are someway connected to each other, there can be not only one but combination of mechanisms involved.

The mechanisms of stress-induced mutagenesis have been investigated mostly in *E. coli*; however, these discoveries are not entirely compatible with other bacteria. The molecular mechanisms of stationary phase mutagenesis in non-enteric bacteria including pseudomonads have remained poorly investigated so far. Our laboratory uses *Pseudomonas putida* as a model system to study mutational processes in starving bacteria. *P. putida* belongs to the group of ecologically widely distributed bacteria capable to adapt rapidly to different environments such as soil and water or plant and animal tissues. Therefore, the study of mutational processes in *P. putida* would provide the knowledge of evolutionary strategies in stressed bacteria. Our pioneering study of stationary phase mutagenesis in *P. putida* indicated that mutational processes in growing and starving *P. putida* must be different (Kasak *et al.*, 1997). Therefore, the aim of the current dissertation was to enlighten the molecular

mechanisms underlying stress-induced mutational processes in soil bacterium *P. putida*. I was particularly interested in the impact of oxidative DNA damage on stationary phase mutagenesis in *P. putida* with the concomitant involvement of oxidative DNA damage defense systems and especially that of oxidized guanine repair. Another goal of the dissertation was to clarify the involvement of one of the major error-avoidance pathways, mismatch repair, in stationary phase mutagenesis in *P. putida*.

1. REVIEW OF LITERATURE

1.1. DNA damage

In the very diverse environments bacteria live, they have to resist with different kind of environmental conditions. The genome of bacteria is therefore continuously challenged by a various number of exogenous and endogenous agents as well as by cellular processes. The damage to genome hinders both the DNA replication and transcription processes. In order to maintain genomic integrity lesions that may arise, have to be removed. In case the lesion-containing DNA is replicated, mutations may emerge.

The sources of mutations are very diverse. Firstly, when DNA is exposed to an outside agent or mutagen then it may lead to the generation of induced mutations. For instance, ionizing radiation (IR) produces a range of effects on DNA such as breaks in one or both DNA strands or damage to nitrogenous bases. Also, ultraviolet (UV) radiation although being less energetic is still mutagenic, is absorbed preferentially by organic compounds and bases of DNA, especially by pyrimidines producing mostly pyrimidine dimers (for instance, thymine dimers). On the other hand, mutations may occur spontaneously as well, for example, as a result of natural processes of cells during DNA replication, recombination and by spontaneous deamination or due to tautomerization. In addition, aerobically growing organisms face the problem of oxidative DNA damage as an important source of mutagenesis. For example, oxidative DNA damage has serious consequences in humans as has been implicated in the normal process of aging, also considered as an important cause of degenerative diseases such as cancer, immune-system decline, cardiovascular diseases and neurodegenerative disorders (Cooke *et al.*, 2003; Wilson and Bohr, 2007). The hazardous effect of oxygen is conferred by reactive oxygen species (ROS) that are generated as byproducts of normal cellular metabolism in respiratory electron transport chains. Alternatively, ROS can be produced also as a consequence of exposure to exogenous factors such as IR or chemical oxidants or free radicals (Friedberg *et al.*, 2004). ROS include singlet oxygen, superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^\bullet$) which can cause different types of DNA damage, many of which are mutagenic (Friedberg *et al.*, 2004). ROS can also oxidize RNA, lipids and proteins (Demple and Harrison, 1994; Imlay, 2003).

As one of the topics of my dissertation concerns the impact of oxidative damage on stationary phase mutagenesis as well as the involvement of oxidative DNA damage defense mechanisms, next chapters of the dissertation are dedicated to the oxidative DNA damage, including its formation and systems for minimizing and repair its hazardous effects. In the second part of the

literature overview I will describe mutational processes in stationary phase bacteria.

1.2. Oxidative DNA damage and defense mechanisms

During evolution, two main pathways have developed: the release of oxygen by photosynthesis and the aerobic respiration. These metabolic systems produce also dangerous molecules for the cells, collectively designated as reactive oxygen species that are constantly formed (Imlay, 2003). Thus, all aerobic organisms from three domains of life face the universal problem of oxygen metabolism as to keep the balance between oxidants and antioxidants.

One of the possible pathways among the processes by which intracellular reactive oxygen species are generated is the partial reduction of molecular oxygen by autooxidation of flavoproteins resulting in production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Imlay, 2003; Massey, 1994). Due to strong anionic charge, superoxide cannot easily react with electron-rich molecules like DNA and amino acids (Imlay, 2003). Nevertheless, superoxide finds good target at metal clusters of proteins. For instance, Fe(II) ions in catalytic center of enzymes bearing 4Fe–4S clusters are susceptible to oxidation (Flint *et al.*, 1993; Liochev and Fridovich, 1994). Importantly, the oxidized clusters are not stable and become degraded, giving the nonfunctional enzyme and release of "free" iron (Keyer and Imlay, 1996, 1997). Latter is available to react with hydrogen peroxide through the Fenton reaction to produce hydroxyl radical, the most hazardous oxygen species as it can oxidize most organic molecules (Imlay, 2003). Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow OH^- + FeO^{2+} + H^+ \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$.

Reaction with ROS leads to oxidative DNA damage such as modified bases and sugars, single- and double-strand breaks in DNA, apurinic/apyrimidinic (AP) sites, DNA-protein crosslinks and clustered damaged sites (David-Cordonnier *et al.*, 2000; Dizdaroglu, 1992, 2005), all of these are invariably mutagenic. In fact, more than 50 different base lesions as products of oxidative damage to DNA have been identified (Cadet *et al.*, 1999). However, among the four nucleobases, the purine nucleobase guanine is the most susceptible to oxidation resulting in the formation of 7,8-dihydro-8-oxoguanine (also named as 8-oxoguanine, 8-oxoG or GO) or its minor tautomer 7,8-dihydro-8-hydroxyguanine along with lesser amounts of a ring-opened product 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (Fapy-guanine) (Burrows and Muller, 1998; Neeley and Essigmann, 2006). 8-oxoG is produced at the G:C base pair within duplex DNA by the reaction of a hydroxyl radical ($OH^{\bullet}$) on the C8 position of 2′-deoxyguanosine (dG) or on the free nucleotide form of guanine in the nucleotide pool (Russo *et al.*, 2004). The most prominent damage

to pyrimidines is the generation of thymine glycol (Bjelland and Seeberg, 2003).

Oxidation due to oxygen metabolism byproducts is investigated because oxidized bases have been shown to be miscoding and formed in great amounts under physiological conditions. For example, 8-oxoG has the capacity to be very mutagenic because of its propensity to mispair with adenine during replication. 8-oxoG when positioned in DNA may cause G:C-to-T:A transversions if not removed before (Shibutani *et al.*, 1991), whereas incorporation of 8-oxo-dGTP opposite adenine nucleotide can lead to A:T-to-C:G transversions (Tajiri *et al.*, 1995). However, it has been estimated that 8-oxoG is only mildly mutagenic *in vivo*, giving rise to less than 10% of G-to-T transversions in bacterial and mammal cells (Cheng *et al.*, 1992; Klein *et al.*, 1992; Moriya *et al.*, 1991; Wood *et al.*, 1990).

In order to counteract with oxidative damage, cells have developed several complex defense mechanisms that include enzymatical removal of ROS, nucleotide pool sanitation, and DNA repair. However, an immediate response to ROS is their detoxification by enzymatical pathways.

1.2.1. First level of defense against ROS

Various defense systems have evolved against ROS. In case the defense systems against oxidants are inefficient, cellular damage increases leading to the situation of oxidative stress.

In order to minimize and repair the damaging effect of oxidative stress, enzymatical mechanisms are used in the first order. Superoxide dismutase (SOD) is the enzyme that converts most of the superoxide (O_2^-) to hydrogen peroxide (H_2O_2) (Fig. 1, reaction 1). SOD genes can be found in aerobic organisms and homologues are found in some anaerobes (Fridovich, 1995). Another possibility for reduction of superoxide to H_2O_2 without molecular oxygen (O_2) byproduct is to utilize superoxide reductase (SOR) (Fig. 1, reaction 2) (Imlay, 2002).

As it is evident from above, superoxide is converted to hydrogen peroxide by enzymatic reactions. Although H_2O_2 itself is a moderate oxidant, its targets include 4Fe-4S clusters of the enzymes as well as sulfur atoms of cysteine and methionine residues (Imlay, 2003). Therefore, there must be mechanisms eliminating H_2O_2 as well. There is an enzyme for decomposition of H_2O_2, called catalase, which general chemical reaction results in production of water and molecular oxygen (Fig. 1, reaction 3). An alternative strategy uses different peroxidases´ activities such as glutathione and thioredoxin-dependent peroxidase, to reduce H_2O_2 to water without the production of molecular oxygen (Fig. 1, reaction 4).

$$2\ O_2^- + 2\ H+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$O_2^- + 2\ H^+ + e^- \rightarrow H_2O_2 \quad (2)$$

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2 \quad (3)$$

$$2\ e^- + 2\ H^+ + H_2O_2 \rightarrow 2\ H_2O \quad (4)$$

Figure 1. Deactivation of reactive oxygen species by superoxide dismutase (1), superoxide reductase (2), catalase (3) and peroxidase (4) (Kurtz, 2004).

Alternatively, in order to alleviate the outcome of oxidative stress, the production of hazardous hydroxyl radicals must be diminished and one of the ways to counteract with it is to decrease the level of "free" ferrous iron (component of Fenton reaction). Although iron is necessary to sustain a wide variety of biological reactions, it may be also potentially toxic element. Therefore, the balanced and complex regulatory system for iron metabolism is developed in organisms. There are mainly two possibilities to minimize the level of "free" iron in cells. First of them is up– or downregulation of iron-uptake systems depending on the iron-deprivation state of the cell (siderophores, transferrin) (Wandersman and Delepelaire, 2004), and second one is up– or downregulation of the iron storage proteins (ferritin) (Theil *et al.*, 2006). In response to H_2O_2, the expression of enzymes that belong to ferritin-like superfamily (ferritin, bacterioferritin) and Dps-like proteins are induced that can scavenge iron ions (Andrews *et al.*, 2003; Baaghil *et al.*, 2003; Ceci *et al.*, 2004; Ilari *et al.*, 2002). Therefore, iron homeostasis is linked to oxidative stress via damage by iron-sulfur cluster resulting in increased level of "free" iron and following increase in hydroxyl radical production by Fenton reaction (Imlay, 2003). However, iron homeostasis is beyond the scope of my dissertation and the focus is on the subject of other oxidative damage defense systems.

1.2.2. Dps-conferred defense against oxidative damage

In addition to the enzyme-mediated removal of oxygen species described above (catalase, superoxide dismutase and peroxidase activities) and the repair of oxidatively damaged DNA by enzymes that belong to the repair systems that will be described below, there is also a third strategy of protection from the harmful effect of ROS – the Dps-mediated DNA crystallization.

The expression of *dps*-encoded Dps protein (DNA binding proteins from starved cells) is activated under nutrient and oxidative stress as one of the stationary phase sigma factor RpoS-dependent genes. This 19 kDa non-specific DNA-binding protein was originally discovered in 3-days old *E. coli* cells as

one of the proteins with the ability to give starvation-induced resistance against hydrogen peroxide (Almiron *et al.*, 1992; Martinez and Kolter, 1997). However, later studies revealed that *dps* is expressed also in growing bacteria in response to low doses of hydrogen peroxide (Altuvia *et al.*, 1994).

Dps is classified as a member of the bacterial nucleoid-associated or histone-like protein family which includes HU (heat-unstable nucleoid protein), H-NS (histone-like nucleoid structuring protein), IHF (integration host factor) and Fis (factor for inversion stimulation) (Grant *et al.*, 1998; Schmid, 1990). Dps associates with nucleoid in a uniform fashion (Almiron *et al.*, 1992; Azam *et al.*, 2000) by further condensing the nucleoid in stationary phase (Kolter *et al.*, 1993). Dps protein constitutes the major component of the chromatin in stationary phase bacteria (Ali Azam *et al.*, 1999). The number of Dps molecules in early stationary phase cells of *E. coli* is estimated to be 120 000 molecules per cell (1 Dps per 40 bp DNA), that is 12-fold higher than in exponentially growing cells and the level of Dps molecules increases gradually in an RpoS-dependent manner reaching a peak of about 180 000 molecules per cell at the stationary phase of growth (Ali Azam *et al.*, 1999; Almiron *et al.*, 1992). Dps is required for long-term stationary phase viability under competitive conditions and nutrient stress (Nair and Finkel, 2004). Also, during stationary phase, Dps protects cells against variety of other stressful factors besides oxidative species, namely, against ionizing and UV radiation, acid, base, thermal extremes and iron and copper toxicity (Choi *et al.*, 2000; Nair and Finkel, 2004).

Dps proteins are widely distributed among bacteria. Dps proteins share sequence similarity and a common overall quaternary structure. Thus, confirming the protection of genomic DNA by this way is a general phenomenon (Ceci *et al.*, 2003; Frenkiel-Krispin and Minsky, 2006; Ishikawa *et al.*, 2003; Kauko *et al.*, 2004; Papinutto *et al.*, 2002; Roy *et al.*, 2004; Zanotti *et al.*, 2002; Yamamoto *et al.*, 2002). Dps has been recently characterized also in archaeal species (Ramsay *et al.*, 2006; Reindel *et al.*, 2002; Wiedenheft *et al.*, 2005).

1.2.2.1. Dps expression in *E. coli*

The transcription of *dps* is regulated by different transcription factors in *E. coli*. In stationary phase, the expression of Dps is controlled by IHF as it binds to the upstream region of *dps* promoter (Altuvia *et al.*, 1994; Jeong *et al.*, 2006). In addition, OxyR and LysR-family transcription factors upregulate *dps* gene expression under oxidative stress (H_2O_2) conditions (Altuvia *et al.*, 1994). However, basal *dps* expression occurs in exponentially growing cells in the absence of stress in an OxyR independent manner (Jeong *et al.*, 2006). The expression and accumulation of Dps protein is induced in transition from the exponential growth phase to the stationary phase as a part of the general stress regulon controlled by stationary-phase specific RNA polymerase sigma factor RpoS (σ^S) (Altuvia *et al.*, 1994; Storz and Imlay, 1999). The level of Dps

protein in *E. coli* is controlled also by proteases (Weichart *et al.*, 2003). Namely, ClpXP protease affects the stability of Dps in the exponential phase of growth and ClpAP is involved in maintaining Dps synthesis during stationary phase more indirectly, probably controlling the regulation of Dps at post-transcriptional level (Stephani *et al.*, 2003; Weichart *et al.*, 2003).

1.2.2.2. DNA protection mechanisms conferred by Dps protein

There are two possibilities of how Dps protects DNA against oxidative stress. First, Dps binds DNA in the sequence-independent manner (Grant *et al.*, 1998) that leads to the co-crystallization of DNA–Dps complexes (Frenkiel-Krispin *et al.*, 2001; Wolf *et al.*, 1999) affording the protection against oxidative species and nucleases. In starved cells, Dps formes well-ordered, multi-layered crystalline structures as seen by electron tomography coupled with time studies (Frenkiel-Krispin *et al.*, 2004). Namely, in *E. coli*, at the onset of starvation, bacterial nucleoid undergoes a conformational change into an assembly of layered toroidal structures consisting of DNA and Dps that act further to promote the formation of subsequent DNA-Dps co-crystals by epitaxial growth (Frenkiel-Krispin *et al.*, 2004). The transition from toroidal structure is promoted by an enhanced binding of Dps to DNA that is mediated by the decrease in concentration of free cations in bacterial cytoplasm during starvation period (Frenkiel-Krispin *et al.*, 2001; Frenkiel-Krispin *et al.*, 2004). Thus, in stationary phase bacteria, chromatin undergoes a reversible transition into tightly packed and ordered structures in which DNA is structurally protected (Minsky *et al.*, 2002). This process is dictated by intrinsic properties of DNA (negatively charged) and by starvation-dependent changes in the intracellular energy balance which promote the formation of chromatin toroids further stabilized by binding of Dps (Frenkiel-Krispin *et al.*, 2004).

Despite the lack of apparent sequence similarity, Dps is still considered as a structural homologue of iron storage protein, ferritin, as both of them form multimeric protein shell even though almost all ferritins are 24-mers while Dps is dodecamer (12-mer) (Grant *et al.*, 1998; Hempstead *et al.*, 1997; Theil *et al.*, 2006). Therefore, other part of protection against oxidative species by Dps is conferred by its Fe-chelating activity. The Dps proteins studied have a ferroxidase center in which Fe^{2+} ions and H_2O_2 are detoxified (Grant *et al.*, 1998; Ilari *et al.*, 2000; Ilari *et al.*, 2002; Zhao *et al.*, 2002). Thus, the main ways of how Dps affords the protection of DNA from oxidative assault are by physically associating with DNA and diminishing the possibility of hydroxyl radical production during the time of oxidative stress.

1.2.2.3. Phenotypic effect of Dps on mutagenic processes

Oxidative stress is characteristic to stationary phase bacteria. Under oxidative and nutrition stress conditions Dps protein is expressed and it was shown to afford the protection against ROS (Almiron *et al.*, 1992). Therefore, generation of mutations due to active oxygen species is expected to be lower in cells expressing Dps protein. It was demonstrated by Martinez and Kolter (1997) that when Dps was non-functional then the frequency of mutations induced by hydrogen peroxide was increased *in vivo* (Martinez and Kolter, 1997). Namely, the effect of Dps on mutagenesis was measured by using *E. coli* tester strains containing *lacZ* mutations that allow detection of possible base substitutions (Cupples and Miller, 1989). The frequency of Lac^+ G:C-to-T:A base substitution mutations was approximately 10-fold increased in the presence of H_2O_2 in *dps*-deficient *E. coli* comparing to wild-type bacteria (Martinez and Kolter, 1997). Moreover, Dps overexpression from inducible plasmid in mutator strains lacking either MutM or MutY activity suppressed the mutator phenotype demonstrating its ability to reduce spontaneous base substitutions generated as a result of oxidative species. However, whether Dps could obviate the mutations from occurring also in physiological conditions of bacteria is not investigated so far.

1.3. DNA repair pathways

Besides the activity of ROS detoxifying enzymes and Dps-mediated defense described above, another level of protection against different hazards is provided by DNA repair pathways. In order to ensure genomic integrity, all organisms have developed complex repair pathways and enzymes acting in these systems are conserved from bacteria to humans (Friedberg, 2003; Modrich and Lahue, 1996; Modrich, 2006; Sancar and Reardon, 2004; Slupphaug *et al.*, 2003; Tainer *et al.*, 1995). The various DNA repair pathways can be categorized according to the mechanism of action process, being either damage reversal, damage removal or damage tolerance. The damage reversal category comprises simple enzymatic systems, the ones that act without breaking DNA backbone. For example, photolyase that can bind specifically and with high affinity to the UV light-induced cyclobutane pyrimidine dimers in DNA using blue light as a cosubstrate to convert the damaged bases (Sancar, 1994, 2003; Sancar, 1990). The damage removal category encompasses wide variety of repair systems, all of them eliminate either damaged or inappropriate base or longer oligonucleotide sequences from DNA. For example, sanitization of dNTP pools, MMR and several excision repair pathways like base excision repair (BER), nucleotide excision repair (NER) are involved. Into the third category of repair mechanisms belong recombinational repair (homologous recombination and

nonhomologous end-joining) and also translesion DNA synthesis. Latter is activated when in some reason the lesion in DNA was not removed by repair mechanisms affording the possibility to synthesize DNA past the damage. Translesion DNA synthesis is conducted by specialized DNA polymerases (Friedberg *et al.*, 2002). Table 1 summarizes the main repair mechanisms known in *E. coli*. From the repair systems of damage removal category cited above, NER enzymes in *E. coli* eliminate mostly bulky distortive DNA damage in 12–13-nt-long oligomer by UvrABC excinuclease from DNA [reviewed in (Reardon and Sancar, 2005; Sancar and Reardon, 2004)]. BER pathway removes damaged bases by DNA glycosylases and MMR repairs DNA replication errors such as mispairs and small insertion/deletion loops in concerted action of mispair recognition and excision complex (Bjelland and Seeberg, 2003; Modrich, 2006). Thus, in case of non-functional repair, the frequency of mutations is elevated. The frequeny of mutations is often increased in bacteria being in stationary state under nonoptimal growth conditions. Both BER and MMR pathways have been implicated in avoidance of mutations in stationary phase bacteria. Therefore, following pages of my dissertation will be covered with the literature overview describing BER and MMR pathways as well as the consequences of their deficiency.

Table 1. Major DNA repair pathways in *E. coli*.

Repair pathway	Participating proteins	Repair specificity
Damage reversal		
Photolyase	PhrA	Photoreversal of UV-induced pyrimidine dimers (Kelner, 1949; Newcombe and Whitehead, 1951; Sancar and Rupert, 1978; Sancar, 2003)
O^6-Alkylguanine-DNA alkyltransferase	AGT (Ada, Ogt)	Stoichiometric repair of guanines with O^6-alkyl adducts (Lindahl *et al.*, 1988; Nieminuszczy and Grzesiuk, 2007; Potter *et al.*, 1987)
Oxidative methyl transferase	AlkB	Oxidative demethylation (Falnes *et al.*, 2002; Kataoka *et al.*, 1983; Sedgwick *et al.*, 2007; Trewick *et al.*, 2002)
Damage removal		
Mismatch repair (MMR)	MutS, MutL, MutH	Repair of postreplicative errors, mispairs, frameshifts (Iyer *et al.*, 2006; Lahue *et al.*, 1989; Lu *et al.*, 1983; Tiraby and Fox, 1973)
Nucleotide excision repair (NER)	UvrA, UvrB, UvrC	Removal of bulky lesions distorting DNA helix, including pyrimidine dimers. Removal of nonbulky lesions such as thymine glycols, 8-oxoguanine (Grzesiuk *et al.*, 2001; Howard-Flanders *et al.*, 1966; Lin and Sancar, 1989; Reardon and Sancar, 2005; Sancar and Rupp, 1983; Van Houten *et al.*, 2005)
Base excision repair (BER)	AlkA, Tag	Removal of alkylated and oxidized bases (Clarke *et al.*, 1984; Krokan *et al.*, 1997; Steinum and Seeberg, 1986)
	Mug, Ung	Removal of deamination products (Gallinari and Jiricny, 1996; Huffman *et al.*, 2005; Lindahl, 1974; Varshney *et al.*, 1988)
	Nth, Nei	Removal of oxidized pyrimidines and purines (Demple and Linn, 1980; Gros *et al.*, 2002; Melamede *et al.*, 1994)
Oxidized guanine repair (GO)	MutM, MutY, MutT	Removal of oxidized purines (David *et al.*, 2007; Michaels and Miller, 1992; Tsai-Wu *et al.*, 1992)
Damage tolerance		
Homologous recombination (HR)	RecA, RecBCD, RecFOR, RecJ, RecN	Repair of single and double-strand breaks or gaps, interstrand crosslinks (Clark and Margulies, 1965; Cromie *et al.*, 2001; Kreuzer, 2005; McGlynn and Lloyd, 2002)
Nonhomologous end-joining (NHEJ)	Ku, LigD	Repair of double-strand breaks, presumably during stationary phase (Aravind and Koonin, 2001; Pitcher et al., 2007a; Weller et al., 2002)

1.3.1. Base excision repair in *E. coli*

Base excision repair (BER) is the major DNA repair mechanism for removal of nonbulky damaged nucleotides and also abasic sites in DNA which can form either spontaneously or by enzymatic cleavage of N-glycosidic bond (Krwawicz *et al.*, 2007; Sung and Demple, 2006). Among DNA bases guanine due to its lowest redox potential is preferably oxidized to various products (Neeley and Essigmann, 2006). Oxidized guanine is most thoroughly investigated and it has the most deleterious effects as it can mispair with both adenine and cytosine making it the most significant DNA lesion that is repaired by BER pathway (Michaels and Miller, 1992; Shibutani *et al.*, 1991; Tchou and Grollman, 1993). In fact, it seems that all known DNA repair pathways are involved in repair of oxidative DNA damage. Namely, although repair of DNA containing oxidized bases proceeds mainly through BER, the involvement of NER, recombination pathways and MMR in avoidance of oxidative DNA damage have also been shown (Bai and Lu, 2007; Gros *et al.*, 2002; Kovtun and McMurray, 2007; Mitra *et al.*, 2001; Slupphaug *et al.*, 2003; Wang *et al.*, 2005). Moreover, there are multiple glycosylases with overlapping specifities for oxidized bases (Krokan *et al.*, 1997). Thus, given the various possibilities of complementation in the repair of oxidative base damage, it has been thought to explain the small effects of the absence of single DNA glycosylase in eukaryotes and therefore BER has not received so much attention as MMR or NER, which defects have been linked to variety of diseases (Mitra *et al.*, 2001).

Recent years with overwhelming biophysical and biochemical studies have opened new insights into the processes of damage recognition and subsequent reactions by BER enzymes. Following pages describe BER pathway and the enzymes that participate in it with special emphasis on repair of oxidized guanine as well as its role in spontaneous mutagenesis.

1.3.1.1. DNA damage recognition and reaction path of DNA glycosylases

As the name implies, the initial step in base excision repair is the removal of a base. BER is initiated by abundant DNA glycosylases. They have been shown to bind primarily to lesion-containing DNA strand while the damaged base is being extruded from the duplex DNA and inserted into an extrahelical recognition pocket on the enzyme (Banerjee *et al.*, 2006; Fromme *et al.*, 2004b; Huffman *et al.*, 2005). All the glycosylases studied, bind to the minor groove of DNA, kink DNA at the site of damage, and flip the lesion base out of the DNA major groove. Thus, the initial step of damage recognition involves the deformability of DNA at the base pair destabilized by the presence of lesion. Glycosylases are damage-specific, as only those bases that can be accommodated in a binding pocket upon nucleotide flipping provide necessary contacts and orientation for base excision (Huffman *et al.*, 2005).

The structure and mode of action of many glycosylases have been studied (Parikh *et al.*, 1997). Each of them is programmed to recognize and act upon damaged bases that do not cause major helix distortions locating them from majority of normal bases. Most of the DNA glycosylases remove several structurally different damaged bases while some have very narrow substrate specificities (Huffman *et al.*, 2005; Krokan *et al.*, 1997) (Table 2). In general, the damaged nucleobases, and in some cases, mismatched bases are removed by DNA glycosylases by cleaving the N-glycosidic bond between the base and deoxyribose and the resulting free base is then released, leaving an abasic site (apurinic/apyrimidinic, AP) in the duplex DNA that is further processed by the BER machinery to reconstitute the original undamaged DNA sequence (Barnes *et al.*, 1993; David and Williams, 1998). Each subsequent step in BER produces another form of DNA damage such as AP sites that are cytotoxic and mutagenic, and must be further processed (Gentil *et al.*, 1992). AP sites have miscoding properties due to preferential insertion of adenine opposite to AP site (Strauss, 1991).

BER pathway can be divided into series of sequential steps. DNA glycosylases carry out both lesion recognition and subsequent excision (Fromme *et al.*, 2004b). BER pathway reactions in *E. coli* involve the following steps:

1. spontaneous or enzymatic cleavage of the N-glycosidic bond between target base and deoxyribose leaving the AP site;
2. cleavage of the DNA strand at the AP site;
3. action of the DNA polymerase I by inserting a template nucleotide at the position of the AP site;
4. ligation of the remained nick in the DNA.

The enzymes catalysing the reactions mentioned above are DNA glycosylases, AP endonuclease, DNA polymerase I and DNA ligase. The steps of BER are conserved from *E. coli* to humans, including archaea.

Table 2. *E. coli* DNA *N*-glycosylases and their substrate specificities.

Protein	Function	Known substrates[a]	Reference
Alkylation damage			
AlkA, Tag	alkylbase-DNA glycosylase	3-methyl purines, 7-methyl purines	(Bjelland *et al.*, 1994; Bjelland and Seeberg, 1996)
		5-formyluracil, 5-hydroxymethyluracil	(Bjelland *et al.*, 1994)
		hypoxhantine	(Saparbaev and Laval, 1994)
Oxidation damage			
MutY	adenine DNA glycosylase	**A**:8-oxoG, **A**:G, **A**:C,	(Michaels *et al.*, 1992a; Michaels *et al.*, 1992b)
		2-OH-A:G**, G:**8-oxoG	(Hashiguchi *et al.*, 2002; Zhang *et al.*, 1998)
MutM (Fpg)	oxidized purines DNA glycosylase, formamidopyrimidine DNA glycosylase	**8-oxoG**:C, **8-oxoG**:G, **8-oxoG**:T	(Tchou *et al.*, 1991)
		FapyG:C, **FapyG**:G, **FapyG**:T, FapyAde	(Breimer, 1984; Chetsanga and Lindahl, 1979)
		uracil glycol, 5-formyluracil	(Purmal *et al.*, 1998; Zhang *et al.*, 2000)
		5-hydroxycytosine, 5-hydroxyuracil	(Hatahet *et al.*, 1994)
		thymine glycol, 5,6-dihydrothymine	(D'Ham *et al.*, 1999)
		hydantoins - Gh, Sp opposite G, C, T, A	(Leipold *et al.*, 2000)
		oxazolone, oxaluric acid	(Duarte *et al.*, 2000; Duarte *et al.*, 2001)
Nth (EndoIII)	oxidized pyrimidines DNA glycosylase	thymine glycol, 5,6-dihydrothymine	(D'Ham *et al.*, 1999; Demple and Harrison, 1994)
		6-hydroxy-5,6-dihydrothymine, urea	
		5-hydroxycytosine, 5-hydroxyuracil	(Hatahet *et al.*, 1994)
		uracil glycol, dihydrouracil, 5-formyl-luracil	(Dizdaroglu *et al.*, 1993; Zhang *et al.*, 2000)
		8-oxoG:G, **8-oxoG**:A, Fapy-Ade	(Dizdaroglu *et al.*, 2000; Matsumoto *et al.*, 2001)
		oxazolone, oxaluric acid	(Duarte *et al.*, 2000; Duarte *et al.*, 2001)

Protein	Function	Known substrates[a]	Reference
Nei (EndoVIII)	oxidized pyrimidines DNA glycosylase	thymine glycol, 5,6-dihydrothymine, *β*-ureidoisobutyric acid, urea, AP-sites	(Melamede *et al.*, 1994)
		5-hydroxycytosine, 5-hydroxyuracil, 5-formyluracil	(Jiang *et al.*, 1997b; Zhang *et al.*, 2000)
		8-oxoG:G, **8-oxoG**:A, **8-oxoG**:C, FapyAde	(Dizdaroglu *et al.*, 2001; Hazra *et al.*, 2000)
Deamination products			
Uracil DNA glycosylase (UDG) superfamily			
Ung	Uracil DNA *N*-glycosylase	**U**:G, **U**:A	(Krokan *et al.*, 1997; Lindahl, 1974)
Mug	Mismatch-specific-uracil-DNA glycosylase	**T**:G, **U**:G,	(Barrett *et al.*, 1998; Gallinari and Jiricny, 1996)
		Exocyclic DNA adducts: 3,N^4-ethenocytosine	(Saparbaev and Laval, 1998)
		1,N^2-ethenoguanine	(Saparbaev *et al.*, 2002)

[a] In mismatches, target base is on the left and marked also in bold.

1.3.1.2. Classification of BER DNA glycosylases

Each organism from *E. coli* to humans has diverse collection of DNA glycosylases that recognize one or a small subset of lesions. It is possible to classify glycosylases differently, based either on their substrate specificity, cleavage mechanism (mono– or bifunctional) or according to their three-dimensional structures (Fromme *et al.*, 2004b).

Firstly, according to the crystal structures of several DNA glycosylases solved already, it is possible to classify DNA glycosylases into structural families by their architectural folds (Huffman *et al.*, 2005). Namely, glycosylases can be divided into two structural families according to the presence or absence of a motif: helix-hairpin-helix (HhH) and helix-two-turn-helix (H2TH) family. Representative crystal structures are determined for both families. For example, *E. coli* MutY and Nth belong to the HhH family (Bruner *et al.*, 2000; Fromme and Verdine, 2003; Guan *et al.*, 1998; Kuo *et al.*, 1992), but bacterial MutM/Fpg and Nei carry the H2TH motif (Fromme and Verdine, 2002; Gilboa *et al.*, 2002; Serre *et al.*, 2002; Sugahara *et al.*, 2000).

Secondly, according to the cleavage mechanism distinction can be made between monofunctional DNA glycosylases that lack associated AP lyase activity and bifunctional DNA glycosylases that possess AP lyase activity (Au *et al.*, 1988; Fromme *et al.*, 2004b). Monofunctional DNA glycosylases, such as AlkA, cleave the N´-glycosidic bond by hydrolytic mechanism and then protect AP site until acted upon by an AP endonuclease (Sun *et al.*, 1995; Yamagata *et al.*, 1996) (Fig. 2, the path on the right). *E. coli* AP endonucleases are damage-inducible endonuclease IV (Endo IV, Nfo) and constitutively expressed exonuclease III (Exo III, Xth) (Bailly and Verly, 1989; Mol *et al.*, 2000). Exo III was originally characterized as an exonuclease but later showed to be a multifunctional enzyme with probable primary function as a 5´ AP-endonuclease (Mol *et al.*, 1995). These enzymes hydrolyze the DNA phosphodiester backbone: cleavage occurs on the 5´ side of the AP site leaving 3´-OH and 5´-deoxyribosephosphate (dRP) termini. Obviously, free 3´-OH is a substrate for DNA repair polymerase but after the missing base is replaced, DNA cannot be ligated unless 5´-dRP is not removed before. It has been shown that *E. coli* Nei, MutM/Fpg, RecJ and Exo I enzymes have the ability to excise the 5´-terminal dRP from the Nfo-preincised AP site (Dianov *et al.*, 1994; Graves *et al.*, 1992; Jiang *et al.*, 1997b; Sandigursky and Franklin, 1992).

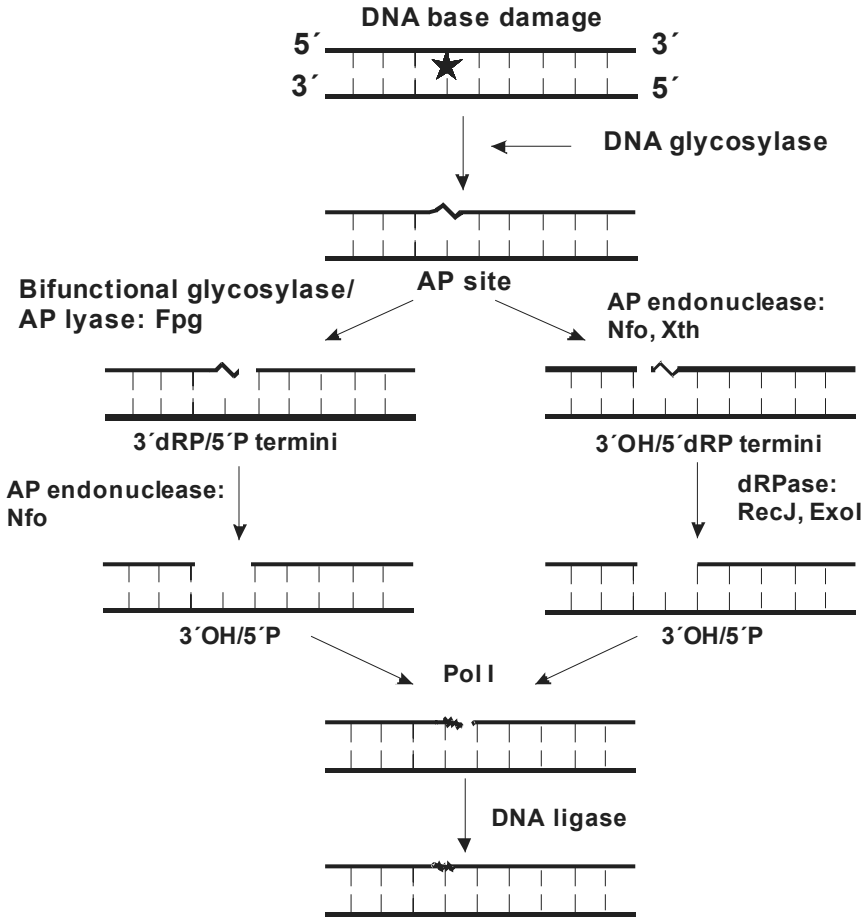

Figure. 2. Schematic outline of the basic steps in base excision repair in *E. coli* involving mono- or bifunctional DNA glycosylase (modified from Mitra *et al.,* 2001). The damaged base is designated with the star (★) in duplex DNA. Details are given in the text.

In contrast, in case of bifunctional glycosylases with associated AP lyase activity such as Nth and MutM/Fpg, AP lyase reaction on the 3´ side of the AP site leaves a gap bordered by 5´-phosphate and 3´-deoxyribosephosphate termini (Krokan *et al.*, 1997) (Fig. 2, the path on the left). Next step needs 3´ phosphatase activity provided by Nfo or Xth to produce a primer for DNA polymerase I. Repair is completed by concerted actions of a DNA polymerase I to fill in the gap and the DNA ligase, to seal the strand (Huffman *et al.*, 2005; Krokan *et al.*, 1997; Seeberg *et al.*, 1995).

Thirdly, DNA glycosylases that remove oxidized base residues can be divided into two functional subgroups: those that repair oxidized purines, for instance *E. coli* formamidopyrimidine DNA glycosylase Fpg or MutM and those that remove oxidized pyrimidines and oxidized purines, for example *E. coli* endonuclease III (Endo III, Nth) and endonuclease VIII (Endo VIII, Nei) (below).

1.3.2. Base excision repair of oxidized purines – oxidized guanine (GO) repair pathway in *E. coli*

It was shown in *E. coli* that the repair of oxidized bases can be attributed to the enzymes that belong to the BER pathway, namely DNA glycosylases MutY (adenine DNA glycosylase) and MutM (Fapy- or formamidopyrimidine, 8-oxoG glycosylase) (Michaels and Miller, 1992; Michaels *et al.*, 1992b). These DNA glycosylases are involved in defence against the mutagenic effects of 8-oxoG (or GO) lesion, which is one of the most stable oxidative product of DNA damage (Michaels and Miller, 1992; Tchou and Grollman, 1993), together with enzyme MutT which specifically eliminates 8-oxodGTP from the nucleotide pool (Maki and Sekiguchi, 1992). As it was shown that 8-oxoG-specific DNA glycosylase is able to excise 8-oxoG from 8-oxoG:C, 8-oxoG:G, 8-oxoG:T mispairs in duplex DNA, it led to the postulation of "GO system" (Grollman and Moriya, 1993; Michaels and Miller, 1992) (Fig. 3). Up to now, the human "GO system" with proteins homologous to those of *E. coli* has been described as well (Slupphaug *et al.*, 2003). According to the GO repair model in *E. coli*, DNA glycosylase MutM removes oxidized guanine opposite cytosine when 8-oxodGTP is incorporated opposite cytosine or when 8-oxoG is formed directly in G:C base pair by ROS (Fig. 3). If 8-oxoG would be left unrepaired, it could pair with adenine during next round of DNA replication and adenine opposite 8-oxoG could be further processed by MutY protein. Also, ROS-induced 8-oxodGTP is often incorporated against adenine (Maki and Sekiguchi, 1992). To alleviate the possible hazardous consequences due to spontaneous mutagenesis that may arise, the third protein known in GO system, nucleoside triphosphatase MutT removes 8-oxodGTP from the nucleotide pool with its pyrophosphohydrolase activity (Maki and Sekiguchi, 1992). MutT hydrolyzes 8-oxodGTP to 8-oxodGMP and pyrophosphate to prevent its use as a substrate by DNA polymerase III (Maki and Sekiguchi, 1992).

The role of GO repair system is to prevent base substitution mutations caused by oxidative DNA damage. The functional DNA glycosylase MutY eliminating adenine from A:8-oxoG mispair diminishes the occurrence of G:C-to-T:A transversions in cell (Moriya and Grollman, 1993; Wood *et al.*, 1990). Similarly, the functional MutM glycosylase removing 8-oxoG from C:8-oxoG

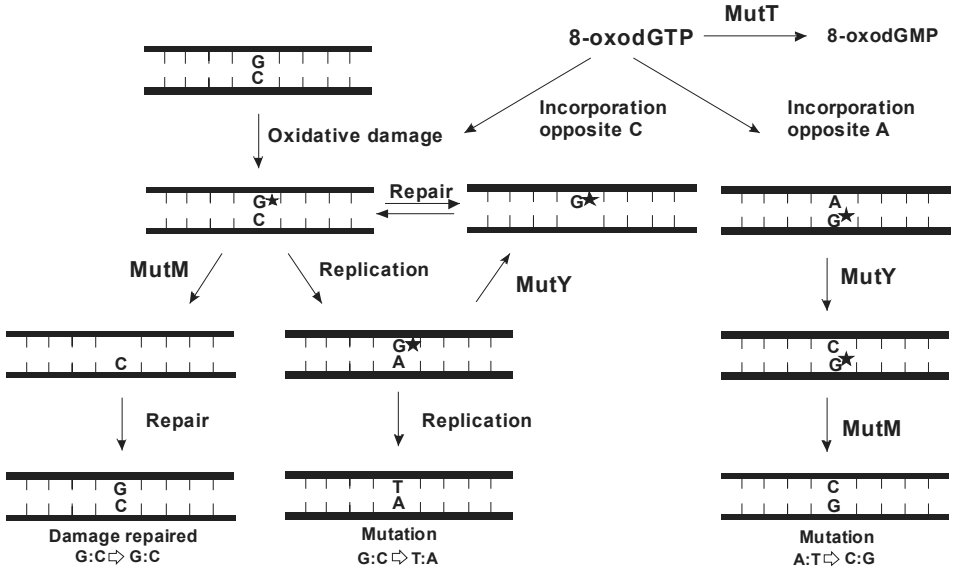

Figure 3. Scheme of GO repair system in *E. coli*. Oxidized guanine (designated as G★) opposite C is a substrate for MutM glycosylase (left path in the scheme). In case C:8-oxoG mispair is not repaired, adenines are frequently added opposite 8-oxoG during DNA replication. When A:8-oxoG mispair is not repaired then it leads to a G:C-to-T:A transversion. MutY removes unmodified adenine when paired opposite 8-oxoG. The excision of adenine from 8-oxoG:A base pair gives the cellular replication machinery an opportunity to insert a cytosine opposite the lesion, which allows this lesion to be removed by MutM. In the right side of scheme: oxidatively damaged guanine nucleotide is removed by MutT enzyme that hydrolyzes 8-oxodGTP to 8-oxodGMP to prevent its incorporation to DNA during replication. 8-oxodGTP can be incorporated opposite cytosine or misincorporated opposite adenine. In latter case, if MutY removes adenine from A:8-oxoG, then A:T-to-C:G mutation will result. Modified from Lu *et al.,* 2001.

mispair avoids G:C-to-T:A transversions because the presence of 8-oxoG in DNA during the replication increases the probability of incorporation of adenine opposite 8-oxoG (Michaels *et al.*, 1991). MutY acts like a safeguard system in case MutM fails to remove the oxidized base before DNA replication proceeds. Over-expression of MutM suppresses the mutator phenotype of MutY eliminating mutagenic 8-oxoG from DNA before DNA replication (Michaels *et al.*, 1992a).

The inactivation of DNA repair genes results in mutator phenotype (Miller, 1996, 1998). It has been shown that spontaneous mutagenesis is increased 20- to 100-fold in *mutY*-deficient *E. coli* cells (Nghiem *et al.*, 1988). The frequency of spontaneous mutation was increased 60- to 140-fold also in *mutY*-defective

pathogenic *Neisseria meningitidis* strain and about 20-fold in *Neisseria gonorrhoeae mutY*-deficient bacteria (Davidsen *et al.*, 2005). Additionally, four *Pseudomonas aeruginosa* mutators isolated from cystic fibrosis (CF) patients had defective *mutY* gene (Oliver *et al.*, 2000). Interestingly, in case of the inactivation of MutM protein activity in *E. coli* the spontaneous mutation frequency was elevated only 10- to 14-fold (Cabrera *et al.*, 1988; Michaels *et al.*, 1992a). Similarly, the *Bacillus anthracis mutM* knockout strain had weaker mutator phenotype than *mutY*-mutant strain (Zeibell *et al.*, 2007). In *E. coli*, the most stronger mutator phenotype is seen in the absence of the MutT activity as the occurrence of A:T-to-C:G transversions is increased 1000-fold compared to wild type (Yanofsky *et al.*, 1966). Thus, oxidized guanine is significant factor of mutagenesis in bacteria but GO system provides an efficient way to alleviate its mutagenic outcome. In the next chapters, the features of MutY and MutM DNA glycosylases will be described more thoroughly.

1.3.2.1. Function and reaction mechanism of adenine DNA glycosylase MutY

E. coli MutY adenine DNA glycosylase was originally identified as an enzyme that prevented G:C-to-T:A transversions (Nghiem *et al.*, 1988; Radicella *et al.*, 1988). The mutator locus was named as *mutY* and the repair activity was found to be independent of the methylation state of the DNA (Lu and Chang, 1988; Radicella *et al.*, 1988). Most important biological substrate for MutY is A:8-oxoG mispair but it can also remove adenine from other base-pairing contexts (Grollman and Moriya, 1993; Michaels and Miller, 1992; Tsai-Wu *et al.*, 1992) (Table 2). MutY has weak guanine glycosylase activity when G is paired with 8-oxoG (Li *et al.*, 2000; Zhang *et al.*, 1998) and therefore may avoid G:C-to-C:G transversions.

E. coli mutY gene encodes an adenine glycosylase of 39 kDa with an iron-sulfur cluster [4Fe-4S] and latter has been proposed to play role in DNA damage sensing (Boon *et al.*, 2003; Michaels *et al.*, 1990; Tsai-Wu *et al.*, 1992). The N-terminal domain (25 kDa) of MutY has the catalytic activity (Gogos *et al.*, 1996; Li *et al.*, 2000; Manuel *et al.*, 1996; Manuel and Lloyd, 1997; Noll *et al.*, 1999). The C-terminal (14 kDa) domain of MutY is important in the recognition of 8-oxoG as the removal of this domain results in loss of discrimination between A:8-oxoG and A:G (Gogos *et al.*, 1996; Li *et al.*, 2000; Noll *et al.*, 1999). The C-terminal domain of MutY has some sequence and structural similarity to 8-oxo-dGTP-hydrolyzing enzyme MutT (Abeyguna-wardana *et al.*, 1995; Noll *et al.*, 1999; Volk *et al.*, 2000).

For now, several crystal structures of different MutY proteins are available. In fact, it was in 1998 when the high-resolution crystal structures of the fully active catalytic core of MutY in complex with adenine revealed that adenine was buried in the active site of the catalytic domain and MutY flipped its target mismatched base completely out of the DNA double helix for glycosylase

action (Guan *et al.*, 1998). Adenine binds in a specificity pocket of MutY but guanine is excluded from the active site. Thus, specific interactions completely envelop the bound extrahelical adenine and preclude binding of other DNA bases (Guan *et al.*, 1998). Further, experiments of stopped-flow fluorescence with MutY excising adenine from its substrate A:8-oxoG confirmed the previous findings and suggested the multiphase reaction profile in which 8-oxoG residue must "flip out" the double-stranded DNA helix before adenine (Bernards *et al.*, 2002). On the contrary to that conclusion, Fromme *et al.* (2004b) suggested on the basis of the crystal structure of MutY obtained in *Bacillus stearothermophilus,* that MutY interacts with DNA in a way that enforces the 55°-bend and extrahelical extrusion of the adenine (Fromme *et al.*, 2004a). Therefore, 8-oxoG lies completely in the DNA helix and 8-oxoG is not "flipped out", but unmodified adenine residue is completely extruded from DNA double helix and inserted into an extrahelical pocket for catalysis reaction of MutY. The specific recognition of oxidatively damaged guanine resides in C-terminal domain of MutY (Gogos *et al.*, 1996; Li *et al.*, 2000; Noll *et al.*, 1999). The unique C-terminal domain contacts with DNA strand containing 8-oxoG lesion, such that 8-oxoG is recognized by the help of hydrogen bond contacts between the enzyme´s N- and C-terminal domains. Therefore, it may be suggested that MutY locates its substrate adenine by recognizing 8-oxoG. However, answer to the question of why MutY preferentially recognizes 8-oxoG versus thymine as the base-pairing partner of adenine remains a challenge for further studies.

1.3.2.2. Function and reaction mechanism of 8-oxoG glycosylase MutM

The 30.2 kDa *E. coli* bifunctional DNA glycosylase/AP lyase MutM is a globular monomer, encoded by the *mutM* gene (Boiteux *et al.*, 1987). MutM was described as a second mutator locus besides MutY that avoids G:C-to-T:A transversions when screened for mutator genes conferring elevated transversion frequencies in *E. coli* (Cabrera *et al.*, 1988). Deficiency in *mutM* gene resulted in an approximately 6-fold increase in the steady-state level of 8-oxoG (Bessho *et al.*, 1992).

Initial studies showed that MutM glycosylase is specifically able to remove broad spectrum of modified purines, for example, imidazole ring-opened form of purine N^7-methylguanine like 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (Fapy-guanine), 4,6-diamino-5-formamidopyrimidine (Fapy-adenine) (Boiteux *et al.*, 1992; Chetsanga and Lindahl, 1979) and mutagenic 8-oxoG residues (Tchou *et al.*, 1991), and a number of pyrimidine products (Hatahet *et al.*, 1994) (Table 2). Furthermore, in addition to removal of oxidized guanine opposite G, T and C, MutM is also able to recognize and remove 8-oxoG secondary oxidized products (Leipold *et al.*, 2000). In addition to its N-glycosylase activity, MutM has also a nicking activity that cleaves both the 5´- and 3´- phosphodiester bonds at an AP site, leaving both the 3´ and 5´ DNA

ends phosphorylated (Bailly *et al.*, 1989). In the AP endonuclease-incised DNA, MutM has 5′ terminal deoxyribosephosphatase (dRpase) activity (Graves *et al.*, 1992).

All DNA glycosylases must recognize their substrate among vast excess of normal DNA. It is not clear yet how do they recognize and search for base damage. However, some light of the subject have shed the intensive biochemical studies of the MutM protein with lesion-containing DNA during the last ten years.

In general, glycosylases have been shown to share common reaction mechanism involving several steps to recognize lesions in DNA. Initial recognition of the damaged base involves bending of DNA at the damaged site and subsequent flipping of the lesion to excise base from DNA helix in order to place it to specific enzyme-pocket (Fromme *et al.*, 2004b; Huffman *et al.*, 2005). The encounter of a substrate by 8-oxoG glycosylase induces distortion and bending of DNA bihelical structure about 60 degrees. While recognizing also cytosine opposite the lesion, MutM protein pushes lesion-containing site out from the DNA double helix (Huffman *et al.*, 2005; Serre *et al.*, 2002). MutM enzyme active site pulls the target base into the 8-oxoG-specific recognition pocket while specific amino acid side chain of MutM fills the position left from pushed-out base therefore stabilizing the distorted structure of DNA.

However, it is not clear how MutM discriminates between guanine and 8-oxoG. In fact, guanine might be recognized occasionally and it would even aid to find adjacent 8-oxoG lesion. As it was proposed, 8-oxoG DNA glycosylase moves rapidly along the DNA double helix probing the ligand into helix to look for substrate base (Banerjee *et al.*, 2006). Normal Watson-Crick base-pairing would not be harmed by such probing event whereas abnormal base pair like 8-oxoG:C would be disrupted. This fast searching process would sometimes recognize guanine instead of 8-oxoG, however, guanine would not be carried to 8-oxoG-specifity pocket but would be placed back to helix. Still, when such occasional event happens, it hinders the forward movement of the enzyme and in case of adjacent 8-oxoG lesions, may lead the enzyme to the lesion site (Banerjee *et al.*, 2006; David *et al.*, 2007).

1.3.2.3. DNA glycosylases for oxidized pyrimidines and purines

There are two DNA glycosylases/AP lyases in *E. coli*, namely, endonuclease III (Endo III, Nth) and endonuclease VIII (Endo VIII, Nei) that recognize specifically pyrimidine-derived lesions in DNA as well as oxidized guanine (Asahara *et al.*, 1989; Cunningham and Weiss, 1985; Cunningham *et al.*, 1989; Gros *et al.*, 2002; Thayer *et al.*, 1995; Wallace, 1998).

The *nth*-encoded endonuclease III (Nth) is a 24 kDa protein with iron-sulfur cluster (Cunningham and Weiss, 1985; Kuo *et al.*, 1992; Thayer *et al.*, 1995), that displays broad substrate specifity for cytosine- and thymine- derived lesions in DNA (Table 2). However, Nth may also remove oxidized purines like

8-oxoG from 8-oxoG:G mispairs and 8-oxoG oxidation products oxazolone and oxaluric acid (Duarte *et al.*, 2000; Duarte *et al.*, 2001; Matsumoto *et al.*, 2001).

Another DNA glycosylase in *E. coli* which specifically recognizes pyrimidine-derived lesions in DNA is Nei or Endo VIII (29.7 kDa) (Jiang *et al.*, 1997a; Jiang *et al.*, 1997b; Melamede *et al.*, 1994; Saito *et al.*, 1997). *E. coli* Nei has strong sequence homology, structural and reaction mechanism similarity to *E. coli* MutM protein although they do not share substrate specificity (Hazra *et al.*, 2000; Jiang *et al.*, 1997b). In addition, although Nei has little or no sequence similarity to Nth protein it is still Nth functional homolog. Nei and Nth proteins have overlapping but not identical substrate specificities for pyrimidine-derived lesions, however, the substrate specificity of Nth is still broader than that of Nei (Burgess *et al.*, 2002; Dizdaroglu *et al.*, 1993; Dizdaroglu *et al.*, 2000; Dizdaroglu, 2005; Jiang *et al.*, 1997b; Zharkov *et al.*, 2002) (Table 2). Still, there is a controversy concerning the substrate specificity of Nei as Blaisdell *et al.* (1999) showed that Nei does not have any significant activity on 8-oxoG:A mispair (Blaisdell *et al.*, 1999). At the same time, Nei could function as a backup enzyme for MutM and MutY proteins as the spontaneous mutation frequency of *mutYmutM* double mutants was increased in *mutYmutMnei* triple mutants. Moreover, when mutational spectra were analyzed, the mutations were G:C-to-TA transversions showing that oxidized guanine is removed in case of MutM and MutY deficiency. However, Hazra *et al.* observed no difference in substrate 8-oxoG preference of Nei among 8-oxoG:A and 8-oxoG:C mispairs (Hazra *et al.*, 2000). Nei was able to remove 8-oxoG from C:8oxoG mispairs, similarly to MutM, and in addition it repaired 8-oxoG when it was misincorporated opposite adenine during the DNA replication (Hazra *et al.*, 2001a). Additional evidence of overlapping activities of endonucleases showed that single *nth* mutants had no increase in spontaneous mutation frequency over wild type (Jiang *et al.*, 1997a). Only modest increase in spontaneous mutation rate (~3-fold) suggested the presence of other activities with similar substrate specificity, probably the activity of Nei. *E. coli* double mutant of Nei^{-} Nth^{-} was hypersensitive to hydrogen peroxide, and rifampicin forward mutation assay showed 20-fold increase in spontaneous mutagenesis compared to wild-type cells (Jiang *et al.*, 1997a), which is comparable to that of *mutM*-deficient strains (~15-fold) (Cabrera *et al.*, 1988; Michaels *et al.*, 1992a).

Recent study showed that there is an interaction between the pathways that are involved in the repair of oxidized purines and pyrimidines, precisely, MutY interacts physically with Nei through its C-terminal domain (Lu *et al.*, 2006). Nei could promote MutY dissociation from its reaction products and acted further on these products. Thus, Nei is able to affect DNA binding of MutY (Lu *et al.*, 2006).

1.4. Nudix hydrolase superfamily

In addition to the elimination of ROS at the enzymatical level, the protection of DNA mediated by Dps and repair of DNA by various DNA repair pathways there is also a fourth line of DNA defense provided at the nucleotide pool level. Namely, the incorporation of damaged bases into DNA is prevented by enzymes that hydrolyze oxidized deoxynucleoside triphosphates (dNTP). These enzymes belong to Nudix hydrolase superfamily. The Nudix hydrolase superfamily consists mainly of pyrophosphohydrolases that hydrolyze substrates that have general structure as nucleoside diphosphate linked to another component, X (NDP-X) to yield NMP plus P-X and the members of the Nudix superfamily can be found in more than 250 species including eukaryotes, archaea, viruses and bacteria (Bessman *et al.*, 1996). The substrates of the Nudix hydrolases include both intact and oxidized (d)NTPs, nucleotide sugars and alcohols, dinucleoside polyphosphates, dinucleotide coenzymes and capped RNA-s; 8-oxodGTP, 2-OH-dATP, which arise from oxidation or other modifications of intact nucleotides (Kamiya, 2003; McLennan, 2006). Thus, some of the Nudix family members sanitize the nucleotide pool and others remove various cellular metabolism endproducts. By hydrolytically removing such compounds, Nudix enzymes protect, regulate and have a signalling role in cellular metabolism (Bessman *et al.*, 1996; Xu *et al.*, 2004).

Substrate catalysis of the Nudix enzymes depends on the conserved 23 amino acids Nudix motif Gx_5Ex_5[UA]xREx_2EExGU, where U is a bulky hydrophobic residue and X is any residue (Bessman *et al.*, 1996; Xu *et al.*, 2004). This motif forms loop-helix-loop structural motif (Koonin, 1993) and the Glu residues in the core of the motif, REx_2EE, have a function as a versatile Mg^{2+}- binding and catalytic site (Abeygunawardana *et al.*, 1995; Gabelli *et al.*, 2001; Kang *et al.*, 2003; Lin *et al.*, 1997). The residues of this motif do not participate in binding of a nitrogenous base but coordinate catalytically essential Mg^{2+} ions. As a consequence, enzymes of the Nudix superfamily hydrolyze a wide range of substrates. Mechanistic diversity and structures of the Nudix enzymes have recently been reviewed in (Mildvan *et al.*, 2005).

In bacteria, there can be various number of Nudix hydrolases. For example, laboratory strains of *E. coli* have 13 Nudix hydrolases, 11 of them are characterized by their enzymatic activities (McLennan, 2006; Xu *et al.*, 2006). There are 26 Nudix hydrolases in *Deinococcus radiodurans* genome whereas only 10 in *Bacillus halodurans* (McLennan, 2006). The first Nudix superfamily protein that was studied genetically and enzymatically was a MutT (NudA) protein of *E. coli* (Bessman *et al.*, 1996). Additional examples of nucleotide pool sanitization and phenotypic effects of *E. coli* Nudix hydrolases such as Orf135 (NudG) and Orf17 (NudB) as well as MutT itself are given in the next chapter.

1.4.1. Nucleotide pool sanitization

The nucleoside triphosphate pyrophosphohydrolase MutT from *E. coli* is a monomeric 15 kDa protein that catalyzes hydrolysis of nucleoside- and deoxynucleoside-triphosphates to yield nucleotide and pyrophosphate. MutT enzyme was the first protein found to degrade mutagenic oxidized DNA precursors, its target was oxidized dGTP (Bhatnagar *et al.*, 1991; Maki and Sekiguchi, 1992). 8-oxodGTP is hydrolyzed by MutT as much as 200-fold more efficiently than dGTP (Bessman *et al.*, 1996; Maki and Sekiguchi, 1992; Xia *et al.*, 2005). As a result, it is widely accepted that the role of MutT is to sanitize the nucleoside triphosphate pool by removing 8-oxodGTP which incorporation to DNA would have mutagenic consequences (Mo *et al.*, 1992; Sakumi *et al.*, 1993; Tajiri *et al.*, 1995). Indeed, inactivation of MutT leads up to ~1000-fold increase in spontaneous mutation frequency because of accumulation of A:T-to-C:G transversions that have formed as a result of incorporation of 8-oxodGTP opposite A (Fowler and Schaaper, 1997; Yanofsky *et al.*, 1966).

MutT protein has both the 8-oxodGTP and 8-oxodGDP hydrolyzing activity as well as activity towards ribonucleotide analogs 8-oxoGTP and 8-oxoGDP (Ito *et al.*, 2005; Taddei *et al.*, 1997a). MutT enzyme has the ability to hydrolyze all above-mentioned substrates and therefore prevent the incorporation of oxidized guanine-containing precursors during DNA and RNA synthesis. Hence, MutT is often referred to as a member of the GO repair system.

In addition to MutT, it seems that three other proteins in *E. coli* also contribute to the elimination of 8-oxodGTP. Firstly, Kobayashi *et al.* (1998) demonstrated that *E. coli ribA*-encoded GTP cyclohydrolase II possesses a pyrophosphatase activity for 8-OH-dGTP and 8-OH-GTP with a higher rate than for GTP whereas dGTP was not hydrolyzed at all (Kobayashi *et al.*, 1998). Since the intracellular concentration of 8-OH-dGTP is low, it seems that the role of RibA protein to control the mutation frequency in *E. coli* cells may be rather limited. However, the *ribA* gene product has antimutagenic effect when over-expressed in *mutT*-deficient bacteria (Kobayashi *et al.*, 1998). Therefore, RibA may act as a backup enzyme of MutT.

Notably, another Nudix family hydrolase protein, Orf135 (NudG), hydrolyzes 8-oxo-dGTP *in vitro* as well (Kamiya *et al.*, 2001). Moreover, *E. coli* Orf135 protein has hydrolyzing activity towards 2-OH-dATP (Fujikawa and Kasai, 2002; Kamiya *et al.*, 2001; O'Handley *et al.*, 2001). Thus, Orf135 may also contribute to the avoidance of mutations caused by 2-OH-dATP. Indeed, the lack of the Orf135 protein-activity increases both the spontaneous mutation as well as H_2O_2-induced mutation frequency due to G:C-to-A:T transitions and G:C-to-T:A transversions while over-expression decreased mutation frequency (Kamiya *et al.*, 2003).

Another member of *E. coli* Nudix hydrolases, Orf17 (NtpA, NudB) has been shown to hydrolyze both the triphosphate and diphosphate derivatives of a

deoxyribonucleoside with similar efficiencies (Hori *et al.*, 2005). Specifically, Orf17 hydrolyzes 8-OH-dATP and the corresponding diphosphate 8-OH-dADP even 2.3-fold more efficiently than 8-OH-dATP *in vitro* (Hori *et al.*, 2005). One may speculate that this enzyme may contribute to the prevention of incorporation of 8-OH-dATP into DNA by its dual hydrolysis of both diphosphates and triphosphates. Orf17 was also able to eliminate known MutT substrate 8-oxodGTP *in vivo* (Hori *et al.*, 2006). Therefore, Orf17 could act as a backup enzyme for MutT protein, both of them hydrolyze 8-oxodGTP and its diphosphate derivative (Hori *et al.*, 2005; Ito *et al.*, 2005). In conclusion, MutT acts as a major 8-oxo-dGTP hydrolyzing enzyme although at least three other proteins (Orf17, Orf135, GTP cyclohydrolase II) are able to hydrolyze the mutagenic 8-oxo-dGTP as well.

1.5. Mutagenesis of oxidized deoxynucleoside triphosphates

1.5.1. Mutagenic potential of oxidized adenine

Besides the oxidation of guanine, other bases can be oxidized as well. Among oxidized bases the oxidized adenine has gathered interest. Although lesions of the oxidized adenine are less widespread than those of oxidized guanine, oxidatively damaged adenine lesions such as 7,8-dihydro-8-oxoadenine (8-oxoA) or 2-hydroxy adenine (2-OH-Ade) or Fapy-Ade are still formed. *In vitro* experiments have shown that the formation of 2-OH-Ade upon treatment with Fenton-type reagent is not so efficient as that of 8-oxoG (Kamiya and Kasai, 1995). Interestingly, the amount of oxidized adenine was similar to that of 8-oxoG after oxidant treatment [review in (Kamiya, 2004)]. Therefore, 2-OH-Ade may accumulate in cells and be also mutagenic. The most likely origin of 2-OH-Ade in DNA seems to be its incorporation by DNA polymerases. However, when 2-OH-Ade is localized in DNA, then according to DNA synthesis with synthetic oligodeoxyribonucleotides, predominantly dGTP followed by dATP and dCTP was inserted opposite 2-OH-Ade by *E. coli* Klenow fragment in most sequence contexts (Kamiya and Kasai, 1996). In support of these findings, the studies in *E. coli* have shown that 2-OH-Ade in DNA induced mainly A-to-G and A-to-T, with a lesser extent of A-to-C substitutions, suggesting the misincorporation of dCTP, dATP and dGTP, respectively (Kamiya and Kasai, 1997). Thus, oxidized adenine must be considered as one of the significant mutagenic DNA lesions.

1.5.2. Mutagenesis of secondary oxidation products of guanine

An interesting recently recognized feature of oxidized guanine is, that it is highly susceptible towards further oxidation compared to the parent guanine and several *in vitro* studies have shown that oxidized guanine itself is a "hot spot" for oxidative damage (Leipold *et al.*, 2000; Tretyakova *et al.*, 1999). It may be also the reason why the electrochemical detection and quantitation of 8-oxodGTP from *E. coli* extracts of wild-type and *mutT*–deficient bacteria did not detect the oxidized guanine, independent of whether bacteria had been oxidatively stressed before or not (Tassotto and Mathews, 2002). However, up to now, a variety of DNA lesions are known to form when 8-oxoG is subjected to oxidative conditions (Burrows and Muller, 1998; Kino and Sugiyama, 2001; Luo *et al.*, 2000, 2001; Niles *et al.*, 2001a, b; Tretyakova *et al.*, 1999). The distribution of products depends on the mechanism of action of the specific oxidant involved and the conditions of the reaction. As an example, when peroxynitrite is used, its preferred reaction with 8-oxoG compared to other four nucleobases results in creation of several lesions including cyanuric acid (Ca), imidazolone (Iz) and its hydrolysis product oxazalone (Oz), guanidinohydantoin (Gh), spiroiminodihydantoin diastereomers (Sp1 and Sp2), and oxaluric acid (Oa) (Dedon and Tannenbaum, 2004).

A number of studies have shown the predominance of G:C-to-A:T, G:C-to-T:A and G:C-to-C:G mutations after *in vitro* DNA replication or upon exposure of cells to different kinds of oxidants (Akasaka and Yamamoto, 1994a, b, 1995; Ono *et al.*, 1995; Tretyakova *et al.*, 2000a). As 8-oxoG is not the known inducer of the G:C-to-C:G substitutions, this result referred to the presence of some additional G-derived lesions. Many reports over the last years have addressed this possibility and detailed studies have clarified the role of guanine secondary oxidation products in mutagenesis (Bjelland and Seeberg, 2003; Neeley and Essigmann, 2006; Tudek, 2003). These various hyperoxidized products are potentially mutagenic yielding G-to-T or/and G-to-C or G-to-A mutations *in vitro* (Duarte *et al.*, 2000; Duarte *et al.*, 2001; Gasparutto *et al.*, 1999) and *in vivo* (Henderson *et al.*, 2002; Henderson *et al.*, 2003; Henderson *et al.*, 2005; Neeley *et al.*, 2004).

Particular interest concerning the 8-oxoG secondary oxidation products have gained spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh). Both Sp and Gh are more blocking lesions for DNA polymerase than 8-oxoG and are highly mutagenic in *in vitro* and in cellular systems (Henderson *et al.*, 2002; Kornyushyna *et al.*, 2002). For example, *E. coli* Klenow fragment (exo$^-$) inserts readily dAMP and dGMP opposite Sp and Gh lesions (Kornyushyna and Burrows, 2003). Mutagenesis assays in *E. coli* with single-stranded lesion-containing viral vector DNA have confirmed Sp to be a stronger block for replication than Gh (Delaney *et al.*, 2007; Henderson *et al.*, 2003).

One of the important factors influencing the mutagenic potential of any given lesion is the efficiency of its repair and the ability of repair enzymes to sense the correct base-pairing context. It has been reported that recognition and excision of the Sp and Gh lesions in DNA *in vitro* occurred by the BER enzyme endonuclease Endo VIII (Nei) which is usually known to repair oxidized pyrimidines (Hazra *et al.*, 2001b). BER glycosylase MutM which efficiently excises 8-oxoG only when paired with C removes 8-oxoG secondary oxidation products Gh, Sp, Oa and Oz when paired with A, G, C or T in *in vitro* conditions (Duarte *et al.*, 2000; Duarte *et al.*, 2001; Hazra *et al.*, 2001b; Leipold *et al.*, 2000; Tretyakova *et al.*, 2000b). Contrary to that, MutY does not excise an A and/or G opposite Gh or Sp *in vitro* (Hazra *et al.*, 2001b; Leipold *et al.*, 2000). Importantly, 8-oxoG secondary oxidation products including Gh and Sp were not repaired significantly by the MutY enzyme *in vivo* neither (Delaney *et al.*, 2007). Based on the suggestion that lesions which may exist in cells at low level remain undetected because they are repaired, Sp lesion has been detected *in vivo* by mass spectrometry in Nei-deficient chromate-oxidized *E. coli* cells and not in *mutYmutM* double mutant strain (Hailer *et al.*, 2005).

So far, the MutT enzyme, known to hydrolyze the triphosphate of 8-oxodGTP with a specificity 1000-fold higher than dGTP (Kamiya *et al.*, 2004; Maki and Sekiguchi, 1992), has not been reported to hydrolyze triphosphates of the various guanine oxidation products discussed above.

In summary, although only 8-oxoG and Sp have been detected *in vivo* so far, nevertheless, given the abundance of 8-oxoG and its ease to further oxidize compared to G, the existence of many secondary oxidation products of G is rather plausible. Moreover, the high mutagenic potential compared to 8-oxoG and still unclear elimination pathways make secondary oxidation products attractive objects for future studies.

1.5.3. Incorporation of oxidatively damaged nucleotides by various DNA polymerases

The oxidized precursors are misincorporated by DNA polymerase(s) and therefore mispairs are formed – 8-OH-dGTP incorporation opposite A induces A:T-to-C:G transversions and 2-OH-dATP incorporation opposite G leads to G:C-to-T:A transversions (Inoue *et al.*, 1998; Kamiya and Kasai, 1995; Maki and Sekiguchi, 1992).

The damaged nucleotides are believed to be incorporated mainly by the replicative DNA polymerase III in *E. coli* (Kamiya and Kasai, 2000; Maki and Sekiguchi, 1992). However, it has been demonstrated that compared to dGTP, 8-oxodGTP is a poor substrate for several DNA polymerases, including *E. coli* DNA polymerases I and II (Einolf *et al.*, 1998; Einolf and Guengerich, 2001). This suggests that 8-oxodGTP level must be quite high in order to substitute

normal dGTP to a significant extent and therefore to be important in the aspect of mutagenesis.

Additionally, it has been shown that error-prone DNA polymerases may be involved in the oxidative mutagenesis through misincorporation of damaged DNA precursors. This was suggested by *in vitro* experiments using purified human DNA polymerase *η* (Masutani *et al.*, 1999) and archaeal Y-family DNA polymerases originated from *Sulfolobus solfataricus* strains P1 and P2 that exclusively incorporate 8-OH-dGTP opposite adenine in the template DNA and 2-OH-dATP opposite guanine and thymine (Shimizu *et al.*, 2003). The first *in vivo* demonstration of Y-family polymerases in mutagenesis by damaged nucleotides in *E. coli* showed that DNA polymerase IV (Pol IV, encoded by the *dinB* gene) (Wagner *et al.*, 1999) promotes G:C-to-T:A mutations when 2-OH-dATP (not 8-OH-dGTP) was directly introduced into cells (Satou *et al.*, 2005). However, DNA polymerase V (Pol V, encoded by the *umuDC* locus) (Reuven *et al.*, 1999; Tang *et al.*, 1999) had suppressive role as decreased the mutagenesis induced by 2-OH-dATP and 8-OH-dGTP (Satou *et al.*, 2005). Recent study with *E. coli* error-prone DNA polymerase IV also suggests that this polymerase might be involved in mutagenesis caused by incorporation of the oxidized deoxynucleoside triphosphates. Namely, Pol IV predominantly incorporated 2-OH-dATP opposite template G and T, and 8-OH-dGTP opposite template adenine *in vitro* (Yamada *et al.*, 2006).

Therefore, as Y-family DNA polymerases insert damaged nucleotides, the role of oxidized nucleotides in mutagenesis might be even larger than expected. Admittedly, the list of factors important in the oxidative mutagenesis should be expanded by counting also the activity of Y-family DNA polymerases.

1.6. DNA mismatch repair

Every organism ranging from bacteria to higher organisms has to maintain the integrity of its genome. The retainment of genetic information is influenced by the fidelity of DNA replication and the efficiency of different DNA repair mechanisms. DNA replication is a complex process and its accuracy is established by the replication process of high-fidelity DNA polymerases. For example, bacterial DNA replication fidelity has been estimated to be in the range of one error per 10^9–10^{10} nucleotide per cell division (Drake, 1999). Rare polymerization errors that have escaped proofreading activity of replicative DNA polymerase, mostly single base-base mismatches or 1 to 4 unpaired nucleotides in the template strand (deletions) or in the primer strand (insertions), are a challenge for postreplicative DNA mismatch repair system (MMR). The DNA mismatch repair system possesses evolutionarily conserved mechanisms of accurately and efficiently repair the overwhelming majority of DNA damage, thereby ensuring genomic integrity. In addition, MMR also recognizes certain

DNA lesions generated during normal cellular metabolism such as oxidized guanine (Bai and Lu, 2007; Mazurek *et al.*, 2002; Russo *et al.*, 2004; Wyrzykowski and Volkert, 2003) as well as UV photoproducts (Feng *et al.*, 1991).

The proteins involved in MMR have been studied extensively (Hsieh, 2001; Marti *et al.*, 2002; Modrich and Lahue, 1996; Modrich, 1997). The significance of MMR has been established as homologs to the *E. coli* MMR proteins have been found in all studied bacteria as well as in yeast and human MMR system (Marti *et al.*, 2002). According to those studies, the inactivation of MMR either permanently or transiently may result in serious consequences. In latter case, the inactivation of MMR occurs transiently when MMR proteins become limiting during rapid error-prone DNA replication or when cells are in stationary phase and/or stressed. It has been estimated that the genetic inactivation of MMR elevates spontaneous mutation rate 50–1000-fold (Jiricny, 1998; Kolodner, 1996; Modrich and Lahue, 1996; Schofield and Hsieh, 2003). The increased mutation rate provides the genetic variation on which selection can act to enhance the fitness of microbial populations in conditions of stress (Matic *et al.*, 2003). MMR deficiency leads to the elevated rates of base substitution and frameshift mutations and permits recombination between partially homologous sequences between different microbial species (Harfe and Jinks-Robertson, 2000; Matic *et al.*, 2003; Rayssiguier *et al.*, 1989). Moreover, inactivation of human MMR causes hereditary non-polyposis colon cancer and confers predisposition to tumour development (Kolodner and Marsischky, 1999).

MMR has been the subject of intense research and numerous excellent and detailed reviews can be found in literature (Iyer *et al.*, 2006; Jiricny, 1998, 2000, 2006; Kunkel and Erie, 2005). To date, the process of MMR in *E. coli* represents the best characterized system. Main proteins involved in *E. coli* MMR are MutS, MutL, MutH, helicase II (UvrD) with single-strand binding protein (SSB), exonucleases, DNA polymerase III and DNA ligase (Table 3).

MMR – excision/resynthesis system can be divided into four phases as follows:

1. recognition of a mismatch;
2. identification of a nascent strand (strand discrimination);
3. formation of an active repair complex;
4. elimination of the mismatch;
5. resynthesis by DNA polymerase III using parental strand as a template.

Following chapters give an overview of mismatch repair pathway in *E. coli*.

Table 3. Proteins of *E. coli* MMR system and their function.

Protein	Function
MutS	Binds base-base mismatches and 1–4 bp insertion/deletion loops
MutL	Matchmaker, coordinates mismatch recognition with excision repair, activates MutH, loads helicase II onto MutH-generated nick on DNA
MutH	Nicks nascent strand in hemimethylated GATC site at 5′ to G
Helicase II	Loaded by MutS and MutL at nick, unwinds DNA for ssDNA excision
Exo VII, RecJ	Excision of ssDNA by 5′ to 3′ exonuclease activity
Exo I, Exo X, ExoVII	Excision of ssDNA by 3′ to 5′ exonuclease activity
SSB	Involved in excision and synthesis of DNA
β-clamp	Interacts with MutS and MutL, possibly enhances MMR complex loading to sites requiring repair or to the replication fork
DNA polymerase III	Accurate DNA synthesis
DNA ligase	Seals nicks after finishing of DNA synthesis

1.6.1. DNA mismatch repair in *E. coli*

1.6.1.1. Mismatch recognition and repair initiation

E. coli MMR recognizes and repairs all base-base mismatches except C:C mispair, and repairs insertions/deletions up to four nucleotides (Marti *et al.*, 2002; Su *et al.*, 1988). The initial step of the MMR, namely mismatch recognition, is conferred by 95 kDa dimeric MutS protein (Jiricny *et al.*, 1988; Su and Modrich, 1986). Insights into mismatch recognition came from crystal structures of *E. coli* MutS bound to different mispairs (Lamers *et al.*, 2000; Natrajan *et al.*, 2003) and *Thermus aquaticus* MutS bound to unpaired thymidine (Obmolova *et al.*, 2000). The structures show striking similarity. MutS monomer consists of five domains: mismatch binding domain (I), connector domain (II), core domain (III), lever-clamp domain (IV) and ATPase domain (V) (Lamers *et al.*, 2000; Obmolova *et al.*, 2000). In all, MutS forms a dimeric clamp-like structure around the heteroduplex. The shape of individual subunit resembles “comma” (Obmolova *et al.*, 2000) and the structure of the dimer is similar to Greek letter θ, with two channels (Hopfner and Tainer, 2000). The heteroduplex DNA is threaded through the larger channel formed by domains I and IV (Lamers *et al.*, 2000; Obmolova *et al.*, 2000; Sixma, 2001). This structure has been named also as a pair of praying hands, with the thumbs folded inwards, and the DNA passing between the fingertips and the thumbs (Jiricny, 2000). The function of the smaller channel is unknown although it may also accommodate a DNA segment (Hopfner and Tainer, 2000). Indeed, it has

been proposed recently that MutS may slide on DNA provided by that smaller channel (Lopez de Saro *et al.*, 2006).

The binding of a MutS dimer to the mispair induces a kink of 60º at the unpaired base towards the major groove. MutS dimer binds to heteroduplex DNA asymmetrically, only one of the two subunits contacts the mispair and this is mediated by highly conserved N-terminal motif Phe-X-Glu in domain I (Marti *et al.*, 2002). Also, binding affinities to mismatches vary depending on the mismatch and local sequence context. The range of binding affinity to mismatch is 10 to 1500-fold higher over the matched basepair (Gradia *et al.*, 2000; Schofield *et al.*, 2001).

MutS protein must recognize many types of alterations in DNA that are structurally diverse. For example G:T mispair that is most efficienty repaired is the most stable and does not alter the DNA double helix, contrary to C:C mispair that distorts DNA but is poorly repaired (Wang *et al.*, 2003). Insertion/deletion loops distort DNA as well. It has been proposed that sensing for mismatches would be a testing of weakened Watson-Crick hydrogen bonding and base-base stacking interactions by trying to insert the phenylalanine of Phe-X-Glu motif of MutS into the double helix and by inducing a kink at the mismatch site rather than searching for structural features (Marti *et al.*, 2002; Sixma, 2001). However, recent atomic force microscopy studies have emphasized DNA bending as a facilitating feature of the initial mismatch recognition by MutS (Wang *et al.*, 2003). Accordingly, MutS binds to DNA non-specifically and bends it in a search of mismatch. When the mispair is recognized, MutS undergoes conformational change to specific recognition complex in which DNA is unbent and mismatched base is flipping out (Wang *et al.*, 2003).

1.6.1.2. Strand discrimination

After recognition of a mismatch by MutS, downstream repair events will be initiated. The postreplicative MMR must assure on which strand repair begins. Therefore, it is of importance to search for a strand discrimination signal, which in *E. coli* but not in most other bacteria and eukaryotes includes MutH protein bound to a hemimethylated *dam* (GATC) site (Modrich and Lahue, 1996). Namely, newly synthesized DNA at GATC sites is normally methylated on adenines after replication, but because deoxyadenine methylase (Dam methylase) lags behind the replication fork by ~ 2 minutes, the newly synthesized strand is transiently unmethylated (Jiricny, 2006; Modrich and Lahue, 1996). The unmethylated GATC sequence is recognized by 25 kDa MutH endonuclease that incises unmodified GATC site 5′ to the G in a mismatch-MutS-MutL-dependent manner (Au *et al.*, 1992; Hall and Matson, 1999; Modrich and Lahue, 1996). The incision provides a strand break that serves as a signal that directs *E. coli* MMR to the unmethylated strand. The incision by MutH can occur either at 5′ or 3′ to the mispair in the unmodified strand.

Many bacteria and eukaryotes lack the Dam methylase and no functional homolog for MutH endonuclease in other organisms but *E. coli* has been found either. The strand discrimination in those organisms may be directed by strand breaks like the 5′ and 3′ termini of Okazaki fragments in the lagging strand or the 3′ terminus of leading strand (Jiricny, 2006; Lacks *et al.*, 1982). Recently, it was discovered that human MutLα has latent endonuclease activity which resembles the characteristics of *E. coli* MutH in MMR (Kadyrov *et al.*, 2006). The protein motif required for the endonuclease activity and for possible strand discrimination was found also in archaeal and bacterial MutL proteins but lacking from bacteria like *E. coli* that rely on GATC methylation to direct MMR (Kadyrov *et al.*, 2006). Thus, the presence of that motif may be regarded as a fundamental difference in the mode of excision initiation in organisms other than *E. coli* (Dao and Modrich, 1998; Kadyrov *et al.*, 2006).

1.6.1.3. Repair complex assembly

For proper repair activity, MMR proteins have to establish the orientation of the location of the mismatch and strand discrimination signal on the heteroduplex. In both *E. coli* and eukaryotes the communication between mismatch and strand discrimination site on the heteroduplex involves formation of multiprotein complexes containing MutS and MutL proteins [reviewed in (Acharya *et al.*, 2003; Schofield and Hsieh, 2003)]. As MutS recognizes mispair, the function of molecular matchmaker MutL in the MMR system is to make a connection between the recognition of a mismatch and the excision of the mismatch from the strand within which it is contained (Sancar and Hearst, 1993). The formation and stability of multiprotein complexes are modulated by ATP. The main focus in the research field of MMR is concentrated on the role of ATP binding and hydrolysis in the ATP-binding sites of both MutS and MutL as well as on poorly known roles of ADP. However, despite the extensive work done by different research groups over a decade, the exact molecular mechanism of how mismatch recognition complex locates the strand discrimination signal is currently not established. On the basis of available biochemical data three models for signalling in MMR have gained more attention in the literature.

First two models (active translocation model and molecular switch model) state that mismatch recognition signalling of downstream events involves ATP-dependent formation of MutS mobile clamp that leaves mismatch in search of the strand discrimination signal. Specifically, electron miscroscopy studies showed that *E. coli* MutS mediates formation of α-shaped loops on hetero-duplex DNA (Allen *et al.*, 1997). According to active translocation model, MutS and its homologs as well as the corresponding MutS-MutL complex need ATP hydrolysis for bidirectional movement from the mismatch to the strand signal along the helix (Allen *et al.*, 1997; Blackwell *et al.*, 1998; Blackwell *et al.*, 2001). Molecular switch model proposes that MutS initially binds to mispair in ADP-bound state. The binding to mismatch then provokes the ADP-ATP

exchange resulting in formation of MutS-sliding clamps which are free to leave the site of mispair independent of ATP hydrolysis (Acharya *et al.*, 2003; Gradia *et al.*, 1997; Gradia *et al.*, 1999). The repeatedly loaded multiple ATP-bound MutS-sliding clamps interact with MutL and together activate MutH. Admittedly, both models are in concordance with the studies showing that in the presence of ATP MutS dissociates from the ends of linear heteroduplex but remains bound to DNA when both ends of a molecule are blocked by biotin-streptavidin complex (Blackwell *et al.*, 2001). However, neither of the models shows what is the role of MutL in signalling the downstream repair events and how the extent of strand degradation is regulated (Schofield *et al.*, 2001). In addition, according to those models, MutS protein leaves the mismatch but then transduction of the signal of mismatch location to endonuclease remains obscure.

According to the third model, MMR proteins remain bound to the site of mismatch searching for the strand discrimination signal. Some similarity to BER pathway can be seen as binding and action of one enzyme on DNA generates a substrate with changed conformation for the next enzyme of repair pathway. Namely, DNA bending or transactivation model proposes that MutS and MutL remain bound to the mismatch with activation of downstream activities at the strand signal mediated by a DNA bending mechanism (Junop *et al.*, 2001; Schofield *et al.*, 2001; Wang and Hays, 2004) (Fig. 4). Therefore, DNA bending rather than movement of complex along the DNA serves to signal the location of mispair. According to this model, MutS ATPase domain which provides kinetic proofreading function, enhances MMR specificity. Binding of ATP-bound MutS to mispair without ATP hydrolysis activates downstream events. However, when MutS binds to matched DNA then bound ATP is hydrolyzed and MutS is released from the DNA (Junop *et al.*, 2001; Schofield *et al.*, 2001; Selmane *et al.*, 2003). In support of this model, it has been shown that MutL blocks the movement of MutS (Schofield *et al.*, 2001) and MutH activation occurs also when mismatch and GATC site are on the same DNA molecules or on different DNA molecules (*in trans*) (Junop *et al.*, 2001). The DNA bending model has gained experimental support (Wang *et al.*, 2003), but it is unclear, how the orientation of the mismatch and strand discrimination signal is established.

To sum up, the molecular details of MMR signalling are far from being clear and there may be multiple pathways of MMR utilized according to a location of particular mispair in chromosome or when particular mismatch is generated (e.g., logarithmic or stationary phase). In support of the latter, there are 186 dimers of MutS in the cell of *E. coli* growing culture whereas its amount decreases in stationary phase cell (Tsui *et al.*, 1997).

1.6.1.4. Strand excision

The step of strand excision removes the mispair from nascent strand allowing repair synthesis to complete the repair.

In *E. coli*, the unmethylated GATC sequence is recognized by MutH endonuclease that incises unmodified GATC site 5′ to the G (Au *et al.*, 1992; Modrich and Lahue, 1996). The nicking activity of MutH is stimulated in a mismatch-dependent manner by MutS, MutL and ATP (Au *et al.*, 1992). Assembly of MutS-MutL-heteroduplex ternary complex then activates the excision system which involves DNA helicase II (UvrD) and several single-strand specific exonucleases. MutS and MutL activate unwinding activity of DNA helicase II (UvrD) on nicked DNA in a mismatch-dependent manner (Yamaguchi *et al.*, 1998). MutL and UvrD interact physically (Hall *et al.*, 1998) and MutL stimulates the unwinding activity of UvrD (Junop *et al.*, 2003; Mechanic *et al.*, 2000; Yamaguchi *et al.*, 1998). UvrD is able to translocate approximately 40–50 bp in 3′ to 5′ direction before dissociation (Ali and Lohman, 1997). As the mismatch signal can be 1 kb away, MutL may load UvrD productively onto the DNA to assure the proper orientation of UvrD (Mechanic *et al.*, 2000).

Unwounded DNA is stabilized by single-stranded-binding (SSB) proteins. In following, nascent DNA strand comprising mismatch is degraded by exonucleases depending on whether MutH incision occurs 3′ or 5′ to the mismatch. When MutH incision occurs 5′ to the mismatch then single-stranded DNA is degraded by ExoVII or RecJ exonuclease with 5′ to 3′ orientation (Grilley *et al.*, 1993). When MutH cleavage occurs 3′ to the mismatch, excision requires ExoI, ExoVII or ExoX (Lahue *et al.*, 1989; Viswanathan and Lovett, 1999). MMR repair is finished with the action of DNA polymerase III and DNA ligase (Fig. 4).

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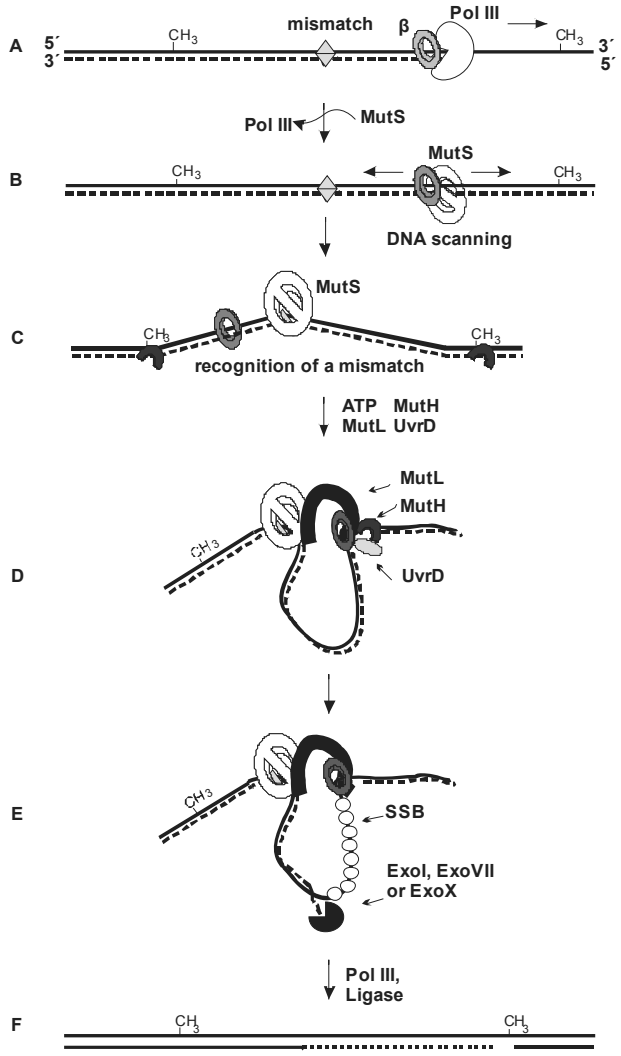

Figure 4. Mechanism of *E. coli* DNA mismatch repair (modified from Junop *et al.,* 2001; Lopez-de Saro *et al.,* 2006). **A**, DNA replication machinery loads β sliding clamp that is used by DNA polymerase III for processivity. **B**, The replicase leaves β clamp temporarily thereby allowing MutS to scan newly synthesized DNA for errors. **C**, Mismatch recognition by MutS results in its conformational change and kinking of DNA at the mispair and releasing of β clamp. **D**, In the presence of ATP, MutL is recruited to the MutS-mismacth complex and together they activate MutH to nick the nascent DNA strand, in case of the current scheme it occurs on the 3′ side of the mismatch. MutL competes with Pol III core for binding to β clamp. The interaction of MutL with β occurs at a single-stranded/double stranded DNA junction. DNA looping structure accounts for the distance between the mismatch and the MutH-generated nick. The nick is the entry point for MutL-dependent loading of helicase II (UvrD). **E**, Dependent on whether MutH incision occurs 5′ or 3′ to the mismatch, the damaged strand is degraded either by 5′ or 3′ exonucleases. In this particular scheme, 3′ exonucleases ExoI, ExoVII or ExoX degrade the strand. The binding of single-stranded-DNA-binding proteins (SSB) stabilizes the parental DNA strand. **F**, DNA polymerase III correctly resynthesizes the strand; DNA ligase seals the nick to complete MMR.

1.6.1.5. MMR protein binding with DNA polymerase β sliding clamp

The *E. coli* DNA polymerase β sliding clamp originally identified as a component of chromosomal DNA replication machine has been described to interact also with several other DNA metabolic proteins, for example with DNA ligase and different types of DNA polymerases participating in DNA repair, replication and translesion synthesis (Lopez de Saro and O'Donnell, 2001; Stukenberg *et al.*, 1991). Interestingly, two components of MMR, MutL and MutS also make contacts with β clamp (Lopez de Saro and O'Donnell, 2001; Lopez de Saro *et al.*, 2006). The interaction of MutL and MutS with β sliding clamp is essential since in case the elimination of the β clamp binding domain from either of the proteins, MMR fails to complete *in vivo* (Lopez de Saro *et al.*, 2006). According to the proposed model, β clamp may target MutS to the sites of DNA replication helping to scan along the DNA for possible mispairs. MutL makes direct contacts with β sliding clamp at a junction between ssDNA and dsDNA, which is a natural substrate of DNA polymerase, and at the same time also the structure present in excision step of MMR (Lopez de Saro *et al.*, 2006). Therefore, MutL may either displace polymerase III core from primer terminus because of competition for a binding site with β clamp or β clamp may bind polymerase III and MutL simultaneously, as has been shown in case of polymerase III and polymerase IV binding to β clamp (Indiani *et al.*, 2005). Indeed, *E. coli* clamp loader protein and different DNA polymerases bind β processivity clamp at the same location suggesting that clamp binding may be competitive and regulated (Lopez de Saro *et al.*, 2003). Moreover, the β clamp-binding motif has been identified in most of the sequenced eubacterial DNA polymerases and MutS proteins (Dalrymple *et al.*, 2001).

One of the possible biological roles of DNA polymerase β sliding clamps and MMR protein interaction may be to guard mismatch repair machinery more rapidly and efficiently to the site required to repair. Additionally, as sliding clamp has certain orientation on DNA, it could help the MMR to discriminate between parental and nascent DNA strand (Jiricny, 1998). Latter does not exclude the unique methylation-state dependent strand discrimination in MMR in bacteria like *E. coli* although, quite a big number of bacteria, for example Gram-positive ones contrary to *E. coli*, lack both the Dam methylase and MutH endonuclease and therefore face the same problem of strand discrimination and endonucleolytic nicking as eukaryotes do (Kolodner, 1996; Malik and Henikoff, 2000; Modrich and Lahue, 1996).

To sum up, it seems that MMR and DNA replication are more tightly coupled than previously assumed. Moreover, as both bacterial and eukaryotic MMR systems interact with replication processivity factors [the bacterial β clamp and its functional homologue in eukaryotes – proliferating cell nuclear antigen (Iyer *et al.*, 2006; Kelman and Hurwitz, 1998)], one could find at least in case of complex interplay between DNA replication and mismatch repair – there is not much difference between prokaryotes and higher organisms at all.

1.6.2. Phenotypic effects of MMR proteins

When an important DNA metabolism protein is non-functional then mutation frequency in bacterial cells is increased to the high level and those bacteria are thereby called mutators (Miller, 1996, 1998). Most powerful mutators are often defective in DNA polymerase III proofreading 3′-to-5′ exonuclease ε-subunit activity and/or MMR (Schaaper, 1988). Temporally high mutation rate may be beneficial, providing new adaptations to the changing environments (Sundin and Weigand, 2007; Taddei *et al.*, 1997b). Mutators that are deficient in MMR have 10- to 1000-fold increased frequency of spontaneous base substitutions and frameshift mutations during growth compared to wild type bacteria (Modrich, 1991). MMR-defective mutators have been isolated both in laboratory experiments (Miller *et al.*, 1999; Sniegowski *et al.*, 1997) as well as from the population of pathogenic and commensal bacteria (LeClerc *et al.*, 1996; Matic *et al.*, 1997; Oliver *et al.*, 2000; Richardson *et al.*, 2002). For example, MMR-defective mutators are found to represent > 1% of isolates within naturally occurring populations of *E. coli* and *Salmonella enterica* (LeClerc *et al.*, 1996). Furthermore, 20% of the strains of widely distributed human opportunistic pathogen *P. aeruginosa* isolated from the lungs of CF patients were mutators too (Oliver *et al.*, 2000). Inefficient MMR was behind this mutator phenotype as the high mutation frequency in 4 strains from the 11 independent *P. aeruginosa* strains was complemented with intact *mutS* gene from *P. aeruginosa* strain PAO1. *P. aeruginosa mutS* mutators were also found in another study of isolated strains from CF patients (Hogardt *et al.*, 2006). However, in contrary, pathogenic bacteria *Haemophilus influenzae* and *Neisseria meningitidis* exhibit low mutation frequencies compared to wild type when MMR is non-functional (Davidsen *et al.*, 2007; Watson *et al.*, 2004).

MMR deficiency lowers the barriers of interspecies recombination (Matic *et al.*, 1996). As an example, interspecies recombination capacity by conjugational and transductional gene transfer between *Salmonella* and *Escherichia* increases ~ 1000-fold in case of *mutS* and/or *mutL*-deficiency and ~20-fold in case of *mutH*-deficiency (Rayssiguier *et al.*, 1989; Stambuk and Radman, 1998). Therefore, *mutS* mutators could potentially act as reservoirs for the horizontal gene transfer. However, such high mutation frequency cannot be long-termed in the evolutionary perspective because deleterious mutations may accumulate (Loewe *et al.*, 2003). Possibly, as there has been shown to be the variability in *mutS-rpoS* intergenic region among enterobacteria, horizontal transfer of *mutS* alleles may rescue bacteria from *mutS* mutator phenotype (Brown *et al.*, 2001; Kotewicz *et al.*, 2003).

In order to investigate mechanisms of spontaneous mutagenesis in *E. coli* MMR mutator strains, specificity of mutation in *mutH*-, *mutS*- and *mutL*-deficient strains have been measured by analyzing the spectra of mutations generated with forward mutation assay system in *lacI* gene (Schaaper and Dunn,

1987; Schaaper, 1988; Schaaper and Radman, 1989; Schaaper, 1993). The composition of the mutation spectrum in MMR-deficient srains revealed both single base frameshifts and base substitutions with the preference of the latter. Among base substitutions, transitions were more pronounced than transversions, whereas among transitions A:T-to-G:C was more frequent than G:C-to-A:T (Schaaper and Dunn, 1987). Another study using *E. coli lacZ* tester strains showed that G:C-to-T:A transversions were increased in *mutL*- and *mutS*-deficient *E. coli* cells both in exponentially growing and stationary phase bacteria (Zhao and Winkler, 2000). Overexpression of MutS protein decreased the rate of G:C-to-T:A transversions in stationary phase *E. coli* tester strain. However, authors of the paper did not see overexpression effects with tester strain measuring frameshift mutations.

Taken together, mismatch repair is important pathway in avoidance of spontaneous mutations both in growing and stationary phase bacteria. Given also the wide spectrum of mutations the lack of MMR can produce, its deficiency contributes to the elevation of mutation rates thereby increasing genetic variation that could possibly alleviate the selective pressure encountred during natural living conditions.

1.7. Stationary phase mutagenesis

1.7.1. General phenomenon of stationary phase mutagenesis

Mutational processes have been studied mostly in actively growing bacteria, e.g., during logarithmic growth of bacterial culture. However, that kind of condition is rare in nature. In their natural habitats bacteria spend most of the time in conditions where they have to cope with various stressful situations such as nutrient starvation, high or cold temperatures, excess of DNA damage, as well as different pH and osmolarity conditions and therefore cell division is arrested by exhaustion of nutrients and the rate of growth is low as the accessible nutrients are consumed more rapidly than become available (Nyström, 2004). It is now widely accepted that mutational processes can take place also in stationary-phase bacteria e.g., in the state when the rate of growth slows down as a result of nutrient depletion and accumulation of toxic products to the growth environment.

It was already in 1960s when Ryan and co-workers showed the emergence of spontaneous mutations in conditions of starvation (Foster, 1992; Ryan *et al.*, 1961). Approximately 30 years later the theory was presented again. Namely, in the conditions of selection the mutants arised giving the cell the ability to grow in these conditions whereas the genes that were not under selection did not bear mutations (Cairns *et al.*, 1988). Hence, the authors suggested that occurrence of mutations was not random but mutagenesis was being “directed” toward genes

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that could allow the survival or in other words, preferably the advantageous or adaptive mutations were generated in conditions of starvation (Cairns *et al.*, 1988). Stahl proposed that stationary phase (adaptive) mutation may occur due to a decline in MMR (Stahl, 1988). However, the assumption of "directed" mutations that arise in conditions of starvation was later a subject of intense debate and in year of 1997 was proved not to be true. For now, the prevailing theory is that stressful conditions increase the mutation rate both in advantageous and in non-advantageous genes (Foster, 1997, 1999a; Torkelson *et al.*, 1997). Nowadays, the term has been called either as "stress-induced" or "adaptive" mutation or "stationary phase" mutation and is defined as the mutation process(es) that may lead to the emergence of mutants that are able to grow in growth-restricting conditions whether or not non-selected mutations are also occurring (Foster, 1999a). Since the term of "adaptive" mutation is mostly used in mutagenesis-studies with favourite test system of *E. coli* strain FC40, I prefer to use more general name, stationary phase mutation, the name given to Ryan´s work, the founder of the phenomenon.

For over a decade it has been searched the answer to the question of mechanism of stationary phase mutations. Nowadays, evidences suggest that bacteria respond to various inconvenient situations by changing gene expression patterns by the induction of different stress response pathways and thus, stationary phase mutation is generated as an induced response to the stress. The key priority of the stress-induced responses is to ensure survival under stressful situation(s) and therefore it is not obligatory to restore original genetic information in those conditions. Indeed, based on the studies in *E. coli*, different stresses can result in the increased generation of mutations in stationary phase by various mechanisms (Kivisaar, 2003; Tenaillon *et al.*, 2004). Just to name a few of these mechanisms, different chemical and physical agents (for instance, ionizing radiation) could alter the DNA structure and lead to replication errors. The accumulation of DNA lesions such as pyrimidine dimers or oxidized guanine, reduces the replication fidelity of replicases by inducing SOS system and activating translesion DNA polymerases for mutagenic error-bypass (Goodman, 2002; Schlacher and Goodman, 2007). However, recent reports suggest that SOS response pathway is activated not only by the direct DNA damage, but various environmental and intracellular signals can trigger the SOS response too [reviewed in (Aertsen and Michiels, 2006)]. The induction of error-prone polymerases results in increased mutagenesis possibly by saturating DNA repair systems in stressed bacteria (Schaaper and Radman, 1989). Additionally, different stresses induce the regulon of stationary phase specific sigma factor RpoS (Hengge-Aronis, 2002) that, among its many functions, downregulates MMR (Tsui *et al.*, 1997) thus leading to higher mutation frequency. It has been suggested that this is useful because it is energy-demanding to keep the system working. However, most of bacteria still retain sufficient level of DNA repair activity (Foster, 2000). The efficiency of DNA repair systems is affected also by

environmental agents (for example, nitric oxide) which can inhibit the activity of DNA repair and through this can elevate mutation rates (Wink and Laval, 1994). Additionally, stressful conditions have shown to induce the translocation of mobile elements thereby increasing the genetic variety and contributing to mutational processes in stationary phase bacteria (Ilves *et al.*, 2001; Shapiro, 2005). Thus, the various mechanisms of stationary phase mutagenesis suggest that under stressful situations there may not be only one pathway but more complex combinations of pathways contribute to stationary phase mutagenesis to overcome the harsh living conditions.

Next chapters dealing with the mechanisms of stationary phase mutagenesis will first focus on the discoveries based specifically on *E. coli* model system strain FC40. Amongst the mechanisms of stationary phase mutation, the following pages will concentrate on the role of mismatch repair system and the involvement of error-prone DNA polymerases. The contribution of oxidative DNA damage to stationary phase mutagenesis will be also discussed.

1.7.2. The mechanisms of stationary phase mutagenesis: lessons from the studies in *E. coli* FC40 model system

There are different test systems to study mutational processes in non-dividing bacteria. Most studies of stationary phase mutagenesis have used starvation for a carbon source or for an amino acid. So far, the best studied test system is based on *E. coli* strain FC40 unable to metabolize lactose and therefore most is known about the mechanisms that produce stationary phase mutations in that particular *E. coli* strain. However, similar observations have been made in other organisms (Foster, 1999a). Several models elucidating the mechanisms of stationary phase mutations in *E. coli* FC40 system are extensively reviewed from different points of view (Foster, 1999a, 2000, 2004, 2005; Hastings and Rosenberg, 2002; Hersh *et al.*, 2004; Rosenberg *et al.*, 1998; Rosenberg, 2001; Roth *et al.*, 2006). Following gives first a brief characterization of *E. coli* FC40 test system and then focuses on the mechanisms of stationary phase mutations discovered in the studies with this particular strain.

The basic system developed originally by John Cairns is as follows. *E. coli* strain FC40 is unable to metabolise lactose (Lac^- phenotype) because the chromosomal *lacZ* gene is deleted. However, it contains an F′ conjugative plasmid bearing a *lacI – lacZ* fusion gene with a + 1 frameshift mutation in the *lacI* coding region (Cairns and Foster, 1991). This strain is Lac^- but reverts readily to Lac^+ at a high rate when lactose is the only energy and carbon source. When bacteria were plated onto plates containing lactose then Lac^+ revertants accumulated constantly during a one-week period (5 days) which showed that the generation of Lac^+ revertants after plating is time-dependent not growth-dependent process (Cairns and Foster, 1991). More specifically, when FC40

cells are plated onto lactose minimal plate, then first visible colonies form after two days of incubation. These second day mutants emerged because of Lac^+ mutations occurred during the time of growing (before the plating). When independent cultures are plated in parallel, the scored number of mutants has a Luria-Delbrück distribution (Luria and Delbrück, 1943). During continued incubation, Lac^+ colonies accumulate onto selective plate and these mutant colonies are generated due to the stationary phase or adaptive mutations occurred on solid media after plating the culture. However, as the mutant *lac* allele is leaky (Andersson *et al.*, 1998) then, to prevent parent strain from growing on minimal lactose medium, the tester strain (10^8) is plated with a 10-fold excess of scavenger cells (10^9) that have unfunctional *lac* operon.

A number of mechanisms of stationary phase mutation in *E. coli* FC40 test system that may distinguish it from growth-dependent mutation will be discussed subsequently. Firstly, it was found that the Lac^+ mutational spectrum collected from stationary phase Lac^+ revertants was different from that of growth-dependent revertants (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994). Specifically, almost all stationary phase mutations were 1-nucleotide deletions in small mononucleotide repeats, whereas the spectra of mutations of growth-dependent mutants were more heterogenous.

Next, differently from growth-dependent Lac^+ mutations, the stationary phase mutations were showed to require the RecABCD-dependent recombination and also *ruvAB* and *ruvC* gene products for double-strand-break-repair (DSBR) (Foster *et al.*, 1996; Harris *et al.*, 1994; Harris *et al.*, 1996). The RuvABC as well as RecF and RecA were required for stress-induced mutations also in *E. coli* strains in which the SOS/LexA regulon was constitutively derepressed thus these proteins were able to contribute to Lac^+ point mutagenesis also in other way than in response to SOS induction (He *et al.*, 2006). Furthermore, Radicella *et al*. (1995) demonstrated that Lac^+ reversion requires the conjugal transfer functions of the F′ plasmid (Radicella *et al.*, 1995), although the question of requirement of actual conjugation is not clear yet (Foster and Trimarchi, 1995a, b; Galitski and Roth, 1995; Godoy *et al.*, 2000). The generation of stationary phase mutations needs the Lac^- allele to be on the F′ plasmid (Radicella *et al.*, 1995). When the *lac* region was chromosomal, then Lac^+ reversion was up to 50-fold less frequent. Moreover, the Lac^+ phenotype might be caused by the activity of stationary phase and general stress response sigma factor RpoS (Layton and Foster, 2003; Lombardo *et al.*, 2004). Deficiency of RpoS has been shown to decrease stationary phase mutation frequency about 90% and this is partly due to the fact that RpoS is a positive regulator of Pol IV (Layton and Foster, 2003). The reduction of adaptive Lac^+ mutations as a result of defective Pol IV has been shown also earlier (McKenzie *et al.*, 2001). Thus, in the Lac system, the stress-induced mutations are specific to stationary phase and are regulated positively by SOS DNA-damage response

and the RpoS starvation- and general stress response whereas both of them upregulate error-prone DNA polymerase Pol IV.

There are two alternative models for stationary phase mutations proposed to underly most of the Lac^+ stationary phase mutations. The mechanisms of stationary phase mutation described above apply for recombination-dependent model (Foster and Trimarchi, 1994; Foster, 2004, 2005; Rosenberg *et al.*, 1994). According to the recombination-dependent model, when *E. coli* FC40 is incubated on lactose then owing to leakiness of mutant *lac* allele enough energy is available for occasional replication initiation at one of the vegetative origins of the episome. Low level of nicking is maintained due to conjugal origin and when the nick is encountered, the replication fork collapses creating the substrate for double-strand-break-repair. During the repair process, DNA synthesis can be erroneous in case the Pol IV is recruited to repair synthesis and Lac^+ mutations are generated when this synthesis occurs in *lac* region. However, it has been proposed also another model for generation of Lac^+ mutants, namely an amplification-dependent model (Andersson *et al.*, 1998; Roth and Andersson, 2004; Roth *et al.*, 2006). According to this model, the $Lac^–$ population initially contains few cells with duplicated *lac* region but as mutant *lac* allele is leaky, the cells further amplify *lac* region and grow slowly on lactose thereby producing small colonies with unstable Lac^+ phenotype. The emergence of true Lac^+ revertants among amplifiers is the more probable the more cells with more *lac* copies per cell there are. When ultimately a reversion event (frameshift) occurs in some *lac* copy then this revertant allele is maintained and so the Lac^+ bacteria eventually possess only single Lac^+ allele. According to the amplification-dependent model, the amplification is the precursor of all Lac^+ colonies (unstable Lac^+ amplifiers, true Lac^+ revertants or both). However, as proposed by Foster (2004), there may exist two pathways, one is mutational process that leads to true Lac^+ revertants during the one-week period and the other is simply an amplification process that creates slowly growing colonies that appear later, after 5 days of incubation.

Stationary phase mutation of FC40 includes also a phenomenon called either as hypermutation, general mutagenesis, transient mutation or general hypermutability. The model for hypermutation was originally proposed by Hall (1990) who argued that stressful conditions may induce a hypermutability state in a small subpopulation of starving bacteria. However, he proposed the mutations to be "directed" towards useful genes and the hypermutators would have died unless getting that good mutation (Hall, 1990). Now, it has been shown that a subpopulation of FC40 Lac^+ cells isolated after incubation on lactose minimal medium carry also non-selected mutations elsewhere in their genomes (Godoy *et al.*, 2000; Hendrickson *et al.*, 2002; Rosche and Foster, 1999; Torkelson *et al.*, 1997). The hypermutability state is transient as high mutation rate is abolished after subsequent testing (Rosche and Foster, 1999; Torkelson *et al.*, 1997). Only 10% of Lac^+ revertants arise from hypermutation subpopu-

lation, the rest of Lac^+ revertants (90%) occur in "normal" population (Rosche and Foster, 1999). The hypermutation state requires the activity of Pol IV (Slechta *et al.*, 2003; Tompkins *et al.*, 2003). Also, it has been shown that MMR is not functional among hypermutators (Rosche and Foster, 1999). So, it has been hypothesized that hypermutator state might be caused by inefficiency of MMR and enhanced activity of Pol IV, which is under control of stationary phase sigma factor RpoS (Layton and Foster, 2003). Admittedly, the variability in mutation rates may be greater than that of arising only from hypermutators and "normal" mutators as there might be many bacterial populations with different mutation rates in the starving bacterial population at any given time.

Similar phenomena from other systems than *E. coli* FC40 provide support for the generality of stress-induced hypermutation as a widespread response in bacteria. For instance, according to the phenomenon of "mutagenesis in aging colonies" (MAC) among natural isolates of *E. coli* from diverse habitats worldwide, Bjedov *et al*. (2003) demonstrated that bacteria increase their mutation rates in response to stress of starvation (Bjedov *et al.*, 2003). Specifically, with a sample of randomly chosen isolates authors showed that MAC phenotype increased remarkably between 1-day- and 7-days-old colonies as a result of carbon source starvation and oxidative metabolism. In one natural isolate with strong MAC phenotype tested, the MAC phenotype was genetically controlled by RpoS and carbon-sensing regulators. Thus, given the similarity of the results obtained in natural isolates of *E. coli* with the discoveries made by using the Lac system of laboratory strain of *E. coli* it may be concluded that the mutagenesis is a common response to stress of starvation in stationary phase bacteria. Moreover, the involvement of stress response protein RpoS both in Lac system and in natural isolates indicates that mutational mechanisms are regulated responses in stressful conditions.

1.7.2.1. Modulation of DNA mismatch repair

There are different mechanisms behind the generation of stationary phase mutations. Several studies with FC40 test system have implied that the efficiency of MMR decreases in stressed or starved bacteria. As stated above, mutations in *E. coli* FC40 strain that give rise to the Lac^+ revertants in starving cells differ from those of growth-dependent Lac^+ mutations in an F′ episome (Cairns and Foster, 1991). Namely, in growing bacteria the Lac^+ phenotype was restored by different-range deletions, duplications and insertions in contrast to the starving bacteria where almost all Lac^+ revertants were generated due to –1 bp frameshifts in runs of small mononucleotide repeats (Foster and Trimarchi, 1994; Harris *et al.*, 1994; Rosenberg *et al.*, 1994). It is known that small deletions and insertions up to four bases are corrected by MMR system in *E. coli*, thus the prevalence of –1 bp deletions among Lac^+ revertants led to the hypothesis of MMR malfunctioning in starving bacteria. Moreover, most of the stationary phase revertants are not heritably MMR-defective (Longerich *et al.*, 1995;

Rosenberg *et al.*, 1998; Torkelson *et al.*, 1997), therefore MMR must be transiently insufficient during the stationary phase mutation. Additional support to the idea that MMR is disabled in stationary phase bacteria was provided by the finding that the spectrum of mutations observed in Lac^+ revertants in starved MMR-proficient cells was essentially the same as in exponentially growing *mutS*- and *mutL*-deficient cells (Longerich *et al.*, 1995). The study conducted by Feng *et al.* (1996) gave further support to the above-mentioned suggestion (Feng *et al.*, 1996). Namely, the direct measurement of cellular amount of MMR proteins indicated the predominance of MutS protein (~186 dimers per cell) over MutL (~113 dimers per cell) and MutH (~135 monomers per cell) in exponentially growing *E. coli* cells (Feng *et al.*, 1996). Interestingly, the amount of MutH was reduced approximately three-fold in stationary phase cells compared to exponentially growing cells, whereas the amount of MutL remained unchangeable in cells being in different growth phases (Feng *et al.*, 1996). The amount of MutS decreased about 10-fold in 2-day old stationary phase cells as well as in nutrient-deprived *E. coli* cells. Moreover, the amount of MutS protein in pathogenic *E. coli* strain O157:H7 was shown to decrease about 26-fold in stationary phase cells in comparison with growing bacteria (Li *et al.*, 2003). It has been shown that the amount of MutS and MutH proteins is downregulated by global stress regulator RpoS (Tsui *et al.*, 1997). The downregulation of MMR activity during stationary phase seems to be a common phenomenon as it occurs also among natural isolates of *E. coli* (Bjedov *et al.*, 2003).

First evidence of mismatch repair modulation according to cell physiology and differentiation was provided in year of 1997 when Harris *et al.* (1997) showed that overproduction of MutL but not MutS inhibited stationary phase mutation in FC40 system (but not during growth), indicating that functional MutL is a limiting factor for MMR specifically in stationary phase bacteria (Harris *et al.*, 1997). The suggestion of occurrence of stationary phase mutations as a result of MMR decline became a subject of hot scientific debate. Foster (1999) claimed that MMR is not less effective in error-correction during prolonged stationary phase than in growing bacteria and that the level of MMR proteins is sufficient for the amount of DNA synthesis that occurs (Foster, 1999b). However, Harris *et al*. (1999) still argued that MMR proteins are limiting during stationary phase mutation but not in active growth conditions (Harris *et al.*, 1999).

In addition, an excess of DNA replication errors can saturate MMR and lead to MMR limitation in stationary phase bacteria (Schaaper and Radman, 1989). The saturation of MMR was also shown in studies with mutagenic base analogue – an introduction of plasmid bearing functional MutL significantly reduced mutations induced by base analogue (Negishi *et al.*, 2002). There is, however, also a possibility that only some of the cells in starving population may have lowered MMR activity and DNA polymerase´s mistakes remain

uncorrected (Foster, 2000; Rosche and Foster, 1999). The increase in mutator rate might be also caused by transcriptional or translational errors in genes for DNA repair proteins themselves. Taken together, although there are several ways of disabling the repair activity, it may be assumed that the expression of MMR in stationary phase bacteria could be adjusted to the level sufficient for repair.

1.7.2.2. The involvement of specialized DNA polymerases in stationary phase mutagenesis

Spontaneous DNA damage occurs continuously and such damage may therefore be responsible for stationary phase mutagenesis (Bridges, 1998). Among many types of DNA damage those that have no coding properties tend to block DNA synthesis when encountered on template strand by replicative DNA polymerase. In response to the stall of replication fork the stress pathway called SOS response is induced and thereby the expression of more than 40 proteins is upregulated in *E. coli* (Courcelle *et al.*, 2001). Among the expressed proteins in *E. coli* there are three DNA polymerases – Pol II, encoded by *polB* gene (Qiu and Goodman, 1997), Pol IV, product of the *dinB* gene (Wagner *et al.*, 1999) and Pol V, product of the *umuDC* operon (Reuven *et al.*, 1999; Tang *et al.*, 1999), so *E. coli* is currently known to possess five different DNA polymerases [reviewed in (Friedberg *et al.*, 2000; Friedberg *et al.*, 2002; Goodman, 2002; Nohmi, 2006; Schlacher and Goodman, 2007; Sutton and Walker, 2001)]. DNA polymerases Pol IV, Pol II and Pol V are called either TLS (translesion DNA synthesis), mutagenic, SOS or specialized DNA polymerases. On the basis of sequence similarity, DNA Pol IV and Pol V belong to the recently designated Y-family and Pol II to B family of DNA polymerases (Yang, 2003). Pol IV and Pol V are structurally similar to other DNA polymerases but the much more opened structure enables them to accommodate damaged bases into their active site thereby allowing to carry out translesion DNA synthesis (Ling *et al.*, 2001). Despite its low fidelity and poor processivity, error-prone DNA polymerases Pol IV and Pol V interact with β processivity clamp of high-fidelity DNA Pol III, and this may possibly allow polymerase(s) switching by recently proposed "tool-belt" model shown with Pol IV, when the replicase itself stalles (Indiani *et al.*, 2005; Sutton and Walker, 2001). Subsequently, specialized DNA polymerases can synthesize short stretch past the replication blocking DNA lesions by catalyzing the insertion of a (in)correct dNMP opposite a lesion depending on the nucleotide preference of particular specialized polymerase. After translesion synthesis, specialized polymerase(s) are switched back to Pol III. These polymerases are regarded as replicating the undamaged DNA (nonsubstrate templates) with low fidelity thus resulting in generation of mutations (Yang, 2003). However, according to Friedberg *et al*. (2002) when particular DNA lesion happens to be a preferred substrate for a specialized polymerase then it is able to copy cognate lesions with high genetic fidelity, that is, by incorporating

the nucleotide that normally pairs with the undamaged version of base (Friedberg *et al.*, 2002).

The induction of specialized DNA polymerases by SOS response in stressed bacteria contributes to stationary phase mutagenesis. Different Y-family polymerases exhibit different lesion bypass ability and hence different mutation spectra. Firstly, DNA Pol II, a B-family enzyme which despite its 3′-to-5′ proofreading activity is involved in error-prone TLS by generating –2 frameshifts (Nohmi, 2006). DNA Pol II was shown to be active under lactose selection conditions in FC40 test system while proofreading-defective Pol II increased episomal stationary phase –1 frameshifts 4- to 6-fold (Foster *et al.*, 1995).

DNA Pol IV, which has the highest expression level upon SOS response among five DNA polymerases (Kim *et al.*, 2001) is the major contributor to the stationary phase –1 Lac^+ frameshift mutations (McKenzie *et al.*, 2001). Bull *et al.* (2001) showed that chromosomal stationary phase –1 frameshift mutagenesis in bacteria carrying F′ plasmid is also Pol IV dependent (Bull *et al.*, 2001). Other studies have shown that overexpression of Pol IV in growing bacteria enhanced frameshifts and also base substitutions (Kim *et al.*, 1997; Wagner and Nohmi, 2000). Pol IV has not demonstrated to contribute significantly to chromosomal mutagenesis in growing cells (Kim *et al.*, 2001; Kuban *et al.*, 2004; Strauss *et al.*, 2000).

Third specialized DNA polymerase, Pol V, is not required for stationary phase Lac^+ frameshift generation (McKenzie *et al.*, 2000). However, Pol V-dependent mutagenesis pathway is described for generation of tryptophan independent mutants as a consequence of G:C-to-C:G transversions in *E. coli mutY trpA23* strain held at tryptophan starvation conditions (Timms *et al.*, 1999). Pol V is also central in promoting SOS-dependent mutagenesis, particularly base substitutions in UV radiation-caused damaged chromosomal DNA sites in growing cells (Schlacher and Goodman, 2007). In addition, Pol V in *S. typhimurium* was able to induce –2 frameshift mutations in response to DNA damaging agent (Kokubo *et al.*, 2005).

The expression of the "growth advantage in stationary phase" (GASP) phenotype during stationary phase relies on new advantageous mutations that confer competitive benefit to cells to take over the population (Finkel, 2006; Zambrano *et al.*, 1993). In fact, all three above-mentioned specialized DNA polymerases – Pol II, Pol IV and Pol V – are important for a long-term evolution and survival by providing competitive fitness advantage during stationary phase in the absence of external DNA-damaging agents known to induce the SOS response (Yeiser *et al.*, 2002). When grown individually, there was no difference in survival patterns between specialized polymerases-deficient *E. coli* strains and wild type during 2-month period. In contrast, when grown in competition with wild type, polymerase-deficient strains could not survive more than 10 days. Taking into account the ability of specialized DNA polymerases to synthesize past various lesions in either error-prone or error-free manner, their

contribution to the generation of genetic diversity in the absence of exogenous DNA damaging agents is thus very likely.

1.7.2.3. Contribution of 8-oxoG to stationary phase mutagenesis

It was already more than ten years ago when Bridges *et al*. (1996) demonstrated that 8-oxoG was generated also in starving bacteria where its production was showed to be 3 times greater than in DNA of growing cells (Bridges *et al.*, 1996). Their research focused on the possible role of 8-oxoG formed in DNA during stationary phase mutagenesis in MutM- and MutY-deficient *E. coli* strains. If 8-oxoG would be generated in starving bacteria then the *trpA23* missense mutations should be elevated in *mutY*-deficent cells. The results showed that few prototrophs emerged in the wild type strain whereas mutation frequency was approximately 20-fold increased in *mutY*-deficient strain under prolonged tryptophan starvation conditions. Most of the mutants were generated due to G:C-to-T:A transversions (Bridges *et al.*, 1996; Bridges and Timms, 1997). Therefore, in the absence of active MutY protein, the presence of 8-oxoG can give rise to G:C-to-T:A transversions in starving bacteria. Surprisingly, *mutM*-deficiency enhanced mutation frequency only slightly in starving bacteria but significant increase in mutation frequency compared to *mutY*-deficient strain was seen in case of double mutant *mutYmutM* strain (Bridges *et al.*, 1996). Overexpression of MutY or MutM was able to suppress the mutation frequency in starvation conditions (Bridges *et al.*, 1996). So, 8-oxoG is formed in starving bacteria and constitutes an important component of stationary phase mutagenesis.

Bridges and Timms (1997) demonstrated also that when *trpA23 mutY*-deficient *E. coli* bacteria were held under amino acid tryptophan starvation conditions then among stationary phase mutations there were in addition to G:C-to-T:A transversions also small in-frame deletions found that led to Trp^+ phenotype (Bridges and Timms, 1997). Latter were found in slowly growing mutant bacteria. Increased frequency of small in-frame deletions (2- to 3-bp) compared to wild type was discovered also among MMR-deficient (MutS-, MutL-, MutH-deficient strains) *E. coli* Trp^+ prototrophs (Bridges and Timms, 1997). Such small in-frame deletions were not detected among Trp^+ prototrophs collected from growing mutant bacteria, therefore, authors proposed the involvement of possible pathway operating in starving bacteria by which persisting mismatches could gave rise to deletions (Bridges and Timms, 1997).

The results of Zhang *et al.* (1998) have also demonstrated the involvement of 8-oxoG in starvation associated mutagenesis. According to their results 8-oxoG was formed in conditions of glycose starvation and led to G:C-to-C:G transversions in the absence of functional MutY protein (Zhang *et al.*, 1998). MutY enzyme was able to remove unmodified guanine from 8-oxoG:G mispair.

The deficiency in GO repair system enzymes leads to the accumulation of G:C-to-T:A transversions because of the formation of A:8-oxoG mispairs. It has

been examined whether the MMR repair could contribute to 8-oxoG mutagenesis. Study with overexpression of MMR repair proteins MutS or MutL in stationary phase *mutM*- or *mutY*-deficient *E. coli* revealed that overexpression of MutS but not MutL protein decreased the rate of *lacZ* G:C-to-T:A transversions both in *mutY*-deficient and *mutM*-deficient *E. coli* (Zhao and Winkler, 2000). Therefore, MutS enzyme is able to recognize and repair A:8-oxoG mispairs. In addition, study of Wyrzykowski and Volkert (2003) has demonstrated that MMR acts on oxidized DNA as correcting base-pairs containing 8-oxoG (Wyrzykowski and Volkert, 2003).

An active oxygen species – singlet oxygen (known to produce 8-oxoG), may also be important in generation of stationary phase mutations (Bridges and Timms, 1998). Specifically, the plasmid encoding genes for carotenoid (singlet oxygen scavenger) synthesis was constructed and introduced into *E. coli* amino acid auxotrophic strains. The presence of carotenoid synthesis-confirming plasmid reduced the rate of appearance of prototrophic mutants approximately 2-fold (Bridges and Timms, 1998). Moreover, the presence of endogenous carotenoids reduced the rate of adaptive Lac^+ reversion mutations approximately 2-fold also in the *E. coli* strain FC40 (Bridges *et al.*, 2001). Therefore, the presented evidences suggest that oxidative DNA damage considerably contributes to generation of stationary phase mutations.

1.7.3. Stationary phase mutation in other bacteria

Stationary phase mutation is not strictly related only to enterobacterium *E. coli* as examples of that phenomenon can be found in other bacteria including *S. typhimurium*, *Bacillus subtilis*, *Pseudomonas* sp., *Mycobacterium.* For example, in case of the Gram positive bacterium *B. subtilis*, it has been shown that multiple mechanisms are involved in generating stationary phase mutagenesis. Namely, the studies with *B. subtilis* have demonstrated that differently from *E. coli lac* system, the generation of amino acid histidine prototrophic revertants did not require the activity of RecA protein (Sung and Yasbin, 2002). However, similarly to *E. coli*, mutations were supposed to be generated as a developmentally regulated response in small hypermutable population of the *B. subtilis* culture. This mutagenesis required the activity of transcription factors ComA and ComK (Msadek, 1999) which are known to control the development of competence. Moreover, using the same chromosomal reversion assay system, the absence of *B. subtilis* DNA Pol IV homolog YgiH decreased the frequency of stationary phase His^+ revertants during a 9-day assay period whereas lack of DNA Pol V homolog YgiW had no effect in stationary phase mutations (Sung *et al.*, 2003). Also, MMR deficiency had impact on generation of stationary phase mutations in *B. subtilis* by increasing the numbers of stationary phase revertants about 3-fold (Pedraza-Reyes and Yasbin, 2004). Differently from *E.*

coli test system where MutL was limiting in stationary phase mutation, MutS was showed to be the limiting component of MMR during the stationary phase mutation in *B. subtilis* test system. Furthermore, the role for *mfd*-encoded transcription repair coupling factor protein (TRCF) or Mfd in stationary phase mutation was proposed in *B. subtilis* as the Mfd-deficient strain had diminished capacity for generation of prototrophic revertants (Ross *et al.*, 2006). However, the mechanism of Mfd-mediated stationary phase mutagenesis is yet to be determined.

Recently, it was discovered that in many prokaryotes there is a repair complex functionally homologous to eukaryotic nonhomologous end-joining (NHEJ) pathway known to be critical to eukaryotic genomic stability as it repairs DNA double-strand breaks (DSB) (Pitcher *et al.*, 2007a; Weller *et al.*, 2002). The bacterial NHEJ pathway is composed of Ku and multifunctional ATP-dependent DNA ligase LigD that is involved in DSB repair during stationary phase and it may have a role in stationary phase mutagenesis. It is of interest as many bacteria that possess a NHEJ repair apparatus proteins spend much of their life cycle in stationary phase of growth. In particular, the importance of NHEJ pathway has been demonstrated in case of endospore-forming *B. subtilis* spores as NHEJ mutant strains are sensitive to IR (Moeller *et al.*, 2007), and similarly in *Mycobacterium smegmatis*, as NHEJ mutant strains are more sensitive to IR and to prolonged desiccation than wild type cells during the stationary phase (Pitcher *et al.*, 2007b). Although DSB repair may be one of the possible mechanisms to generate genetic diversity under selection via stationary phase mutations (He *et al.*, 2006), the role(s) of NHEJ repair pathway in stationary phase mutation processes has not been studied so far. Nevertheless, the discovery of DSB repair by NHEJ in prokaryotes demonstrates that bacterial and eukaryotic molecular processes are more similar than previously thought. Hence, challenge is given to future studies in the evidently more and more complex field of bacterial molecular biology.

The phenomenon of stationary phase mutation has been studied also in the soil bacterium *Pseudomonas putida* (Kasak *et al.*, 1997). However, as the molecular mechanisms underlying stationary phase mutation in *P. putida* are a part of the current dissertation, this subject is mainly discussed in sections of results and discussion.

1.8. DNA mismatch repair and oxidized guanine repair system in *Pseudomonas* sp.

Although the representatives of *Pseudomonas* can be found in various environments, such as soil, water, and in the tissues of plants and humans (Rainey and Moxon, 2000), the mechanisms of MMR and GO repair have not been thoroughly studied in any species of *Pseudomonas*. GO repair system in human opportunistic pathogen *P. aeruginosa* has been characterized by cloning and sequencing the putative *mutY*, *mutM*, *mutT* genes with complementing them in suitable *E. coli* strains; however, no GO repair deficient *P. aeruginosa* strains were constructed and examined (Oliver *et al.*, 2002b). *P. aeruginosa* MutM and MutY proteins were 57% and 53% identical to their *E. coli* counterparts, but differently from *E. coli* MutT homologue, the *P. aeruginosa* MutT was significantly larger (315 amino acids versus 129 amino acids) (Oliver *et al.*, 2002b). Similarly to *E. coli* MutT, the sequence of *P. aeruginosa mutT* gene contains the Nudix motif characteristic to the Nudix family hydrolases.

Among naturally occurring populations of *Pseudomonas*, mutators have been isolated. The high frequency of *P. aeruginosa* mutator strains isolated from CF patients' lungs was found to be caused by defective MMR system and ineffective *mutY* gene, while no alterations in *mutM* and *mutT* genes were determined (Oliver *et al.*, 2000; Oliver *et al.*, 2002b). The work done with *P. fluorescence* in searching the efficient root-tip colonizers revealed that one of the best colonizers was a *P. fluorescence* strain defective in GO repair, namely in *mutY* gene (de Weert *et al.*, 2004).

Also, *P. aeruginosa mutS*, *mutL* and *uvrD* genes have been characterized (Oliver *et al.*, 2002a). The identity of *P. aeruginosa* PAO1 MutS and MutL proteins with *E. coli* K12 MutS and MutL proteins is 60% and 46%, respectively. However, MMR in *Pseudomonas* seems rather different from that characterized in *E. coli*. For instance, despite the high homology, the MMR genes of *P. aeruginosa* did not complement the increased mutation frequencies of the corresponding MMR-defective *E. coli* strains. Moreover, no homologous gene to *E. coli mutH* was found in *P. aeruginosa* genome and no homologous gene to *mutL* was found in *P. stutzeri* (Meier and Wackernagel, 2005; Oliver *et al.*, 2002a). Further evidence in support of differences in MMR systems in *E. coli* and *Pseudomonas* species is the fact that in addition to the lack of *mutH* gene, the genomes of *P. aeruginosa*, *P. stutzeri* and *P. putida* lack also the DNA adenine methylase gene *dam* suggesting that no methylation in GATC sequence occurs in these strains (Meier and Wackernagel, 2005).

Kurusu *et al.* (2000) have reported that incomplete *mutS* gene found in *P. putida* strain 33015 encoded smaller (60 kDa) MutS protein (it lacked ~300 aa from N-terminus) than that of other bacteria (~90 kDa) (Kurusu *et al.*, 2000). However, the incomplete *P. putida* MutS protein was still functional as it was

able to partially complement *E. coli mutS*-defective strain and completely complement *B. subtilis mutS*-defective strain (Kurusu *et al.*, 2000). However, the analyses of genome sequences have revealed the existence of a full-length *mutS* gene in *P. putida* strain KT2440 as well as in other species of *Pseudomonas*. So far, no biochemical studies like those for *E. coli* have been conducted on DNA repair systems in *P. putida*. Thus, given the lack of information about DNA repair systems and their possible linkage to stationary phase mutation mechanisms in *P. putida*, we have dedicated our research to fill this gap by starting genetic examination of the involvement of DNA mismatch repair and oxidized guanine repair pathway in avoidance of stationary phase mutations in *P. putida.*

1.9. *P. putida* assay systems for the study of mechanisms of stationary phase mutations

In order to characterize mutational processes one needs a reliable test system. The test systems to study adaptive or stationary phase mutation mechanisms are designed to mimic the natural growth-limiting environment in which bacteria live, (mostly starvation of an essential nutrient or energy source) and are carried out mainly under conditions of non-lethal selection, meaning that at least most of bacteria are alive and have to mutate in order to form a visible colony. For example, the stationary phase mutation process can be studied when amino acid auxotrophic bacteria are incubated in medium lacking the required amino acid or when bacteria are left to starve as incubated on a carbon source they are unable to metabolize. The most favourite test system to study mutational processes in bacterial populations has used latter variant – starvation for a carbon source and employment of *E. coli* strain FC40. Briefly, this strain contains chromosomal *lacZ* gene deletion and an F´ Lac episome bearing a *lacI – lacZ* fusion gene with a + 1 frameshift mutation in the *lacI* coding region (Cairns and Foster, 1991). Thus, when incubated on lactose minimal media, Lac^+ revertants arise in time-dependent manner. The molecular mechanisms for reversion of *lacI – lacZ* fusion gene have been described [reviewed in (Foster, 2004) and section 1.7.2).

Our workgroup has designed novel experimental test systems to study mutational processes in starving soil bacterium *Pseudomonas putida* (Kasak *et al.*, 1997; Tegova *et al.*, 2004). Historically first of these test systems employing a promoterless *pheBA* operon as a reporter in RSF1010-derived plasmid pEST1414 permits to isolate and characterize mutations that create a functional promoter for the transcription of the *pheBA* genes. The phenol degradation genes *pheB* and *pheA* encode catechol 1,2-dioxygenase and phenol monooxygenase, respectively, and are organized in a single *pheBA* operon (Kivisaar

et al., 1991; Nurk *et al.*, 1991). Wild type *P. putida* plasmid-free strain PaW85 cannot degrade phenol without a functional phenol monooxygenase. Introduction of the *pheBA*- or *pheA*-expressing plasmid into phenol non-degrading *P. putida* strain PaW85 confers this strain the ability to use phenol as a sole carbon source (Kivisaar *et al.*, 1991; Nurk *et al.*, 1991). Therefore, the test system based on the promoterless *pheBA* operon in reporterplasmid pEST1414 selects mutants (Phe^+) that gain ability to grow on phenol minimal media due to mutations that create functional promoter for the transcription of the *pheBA* genes.

The assay system described above enables us to study different types of mutations, e.g., base substitutions, deletions and insertions which all create a functional promoter for the transcription of the *pheBA* operon. However, during the usage of the assay system we noticed that there was one mutational hotspot preferably created among others, referring that some types of mutations could have remained undetected. Thus, a novel set of assay systems was created to study the effects of different genetic backgrounds on the frequency of different types of mutations separately (Tegova *et al.*, 2004). Similarly to the earlier test system, these assay systems take advantage of the fact that *P. putida* wild-type strain PaW85 is unable to grow on medium containing phenol as the only carbon source unless it carries the plasmid-encoded phenol monooxygenase gene *pheA*. These novel assay systems enable to detect the appearance of phenol-degrading revertants (Phe^+), which are generated due to various base substitutions eliminating the stop codons TAG, TAA or TGA introduced into the same position (Leu-22) in place of the CTG codon of the *pheA* coding sequence. Another test system measures reversion of +1 frameshift introduced into the *pheA* coding sequence by insertion of A nucleotide into the ACC codon (Thr-56) (Tegova *et al.*, 2004).

Similar experimental route described with *E. coli* FC40 model system is carried out with *P. putida* strains to generate stationary phase mutations (Cairns and Foster, 1991) (see section 1.7.2). Briefly, approximately 10^8 cells (Phe^-) of *P. putida* strain PaW85 carrying one of the above-described assay systems is plated onto phenol-minimal plates containing phenol as a sole source of carbon and energy. First visible Phe^+ colonies formed after two days of incubation on selective plates and the numbers of mutants among cultures had a Luria-Delbrück distribution, which means that mutations occurred in the growing culture prior to the exposure to the selective agent, i.e. phenol (Kasak *et al.*, 1997). However, Phe^+ colonies that emerged on selective plates on day 3 and later contained mutations that occurred after the cells were plated and are called stationary phase mutations. The numbers of Phe^+ mutants that appeared on phenol-minimal plates during one-week period of starvation were less variable and distribution became Poisson, meaning that mutational processes occurred after plating to the phenol-minimal plates (Kasak *et al.*, 1997). Thus, the generation of Phe^+ mutants after plating is time-dependent process and Phe^+

colonies accumulated onto selective plate due to the stationary phase mutations occurring after plating the culture.

1.9.1. Different mutational processes operate in growing and starving *P. putida*

The pioneering study of stationary phase mutation mechanisms in *P. putida* with the test system employing the promoterless *pheBA* operon as a reporter in plasmid pEST1414 demonstrated that the rate of accumulation of Phe^+ mutants depended on the physiological state of bacteria (Kasak *et al.*, 1997). Namely, the accumulation rate of Phe^+ mutants on phenol-minimal media was higher for those bacteria plated from stationary-phase culture than for those plated from exponential phase of growth (Kasak *et al.*, 1997). The studies excluded possibilities that selective agent (phenol) could have acted as a mutagen or that the high accumulation rate of Phe^+ mutants on phenol-minimal media plated from stationary phase of growth could be caused by higher copy number of plasmid in starving bacteria. Also viable cell counts on selective plates remained constant for bacteria plated from stationary phase of growth (Kasak *et al.*, 1997). Thus, it was assumed that in stationary phase some mutational pathway or process, absent in growing bacteria, might facilitate the generation of mutations.

Most impressive argument that accounts for a difference of mutational pathways in bacteria being in stressful conditions versus bacteria growing exponentially is the difference in the mutational spectrum of mutants collected from respective growth conditions. This was demonstrated already in adaptive mutagenesis experiments with *E. coli* FC40 model system by showing that while a variety of deletions, duplications and frameshifts reverted the Lac^- allele during growth, then Lac^+ mutations that arose during lactose selection were –1 frameshifts in short runs of G´s (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994). Also, earlier study of Prival and Cebula (1992) with *Salmonella typhimurium* found significant differences in the distribution of particular transversions at the *hisG428* locus in revertants arising after prolonged histidine starvation compared to those arising after the growth in the presence of histidine (Prival and Cebula, 1992).

The results of the study with *P. putida* indicated that indeed, there must be some specific mechanism(s) that generates specific mutations in stationary phase cells. Analysis of Phe^+ mutation spectra revealed more straightforward confirmation to that suspection. Several Phe^+ mutants emerging on selective plates on days 2 and 3–7 were collected and DNA sequence (~250 bp) upstream of the *pheBA* operon in plasmid pEST1414 was analyzed (Kasak *et al.*, 1997). The analysis of Phe^+ mutants showed that majority (76%) of the promoters for the transcription of the *pheBA* operon in starving bacteria were created as a

result of a particular point mutation C-to-A located 96 nucleotides upstream from the ATG codon of the *pheB* gene in a potential –10 sequence element, which made this sequence more similar to the *E. coli* RNA polymerase σ^{70}-specific –10 consensus sequence. However, mutants collected from selective plate on day 2 contained mainly different range of deletions (59% deletions from 6 to 45 bp versus 25% C-to-A transversions) with lesser amount of insertions in the sequenced region upstream of the *pheB* gene (Kasak *et al.*, 1997). Notably, insertions and deletions (also like those of 2–23 bp found in starving bacteria) optimized the space between potential –10 and –35 hexamers to 17 or 18 bp (Kasak *et al.*, 1997). Substantial amount of fusion-promoters conferring Phe^+ phenotype were created also as a result of transpositions of two mobile DNA elements – Tn*4652* and IS*1411* upstream of the promoterless *pheA* gene in stationary phase and not in growing cells (Kallastu *et al.*, 1998; Kasak *et al.*, 1997; Nurk *et al.*, 1993). Therefore, as stationary phase Phe^+ mutants had different mutation spectra compared to that of growing bacteria, it strengthened further the assumption that mutation-generation process in starving or stationary phase *P. putida* cells is different from that of growing bacteria.

2. RESULTS AND DISCUSSION

2.1. Aims of the study

In their natural habitats bacteria spend most of their life in everchanging growth conditions where intense competition for limited amount of nutrient resources confers pressure to any advantageous mutation that emerges. Bacterial populations exposed to growth-limiting stress, for example starvation of required amino acid or carbon source, may produce mutations in response to stress that could increase genetic variation in some of the members of bacterial population to achieve a phenotype beneficial for survival. The process of stationary phase mutation provides the ways for bacterial population to overcome growth barriers with enhanced mutation rate. There seem to be multiple molecular mechanisms responsible for stationary phase mutagenesis of bacterial population, and some of these differ from those causing spontaneous mutations in growing cells as evidenced from studies referred in sections of review of literature.

So far, stationary phase mutation mechanisms have most thoroughly studied in model systems based on *E. coli*, but as emerging data from other microorganisms assay systems show, the findings obtained with the favourite research organism of the molecular biology, *E. coli*, cannot be directly extrapolated to other bacteria. Moreover, molecular processes in microorganisms other than *E. coli* seem to share more similarities to higher organisms. Among those bacteria are *Pseudomonas* species that encompass one of the most diverse and ecologically significant group of bacteria (Spiers *et al.*, 2000). The wide distribution of *Pseudomonas* sp. among different habitats refers to great adaptability in different environments. Thus, the basics of that would be important in order to understand the mechanisms of colonization of new habitats or pathogenesis of non-enteric bacteria. There are only few studies of mutagenesis of *Pseudomonas* sp. conducted so far, mainly considering human opportunistic pathogen *P. aeruginosa* strains (Kurusu *et al.*, 2000; Oliver *et al.*, 2000; Oliver *et al.*, 2002a). Notably, the field of stress-induced mutagenesis in *Pseudomonas* species is not intensively explored either. As the mechanisms of stress-induced mutagenesis could provide models for evolutionary strategies of non-enteric bacteria in general, the usage of non-pathogenic and more easily manipulated soil bacterium *P. putida* as a model system to study stationary phase mutagenesis mechanisms is just appropriate. Thus, the work of our research group has focused on elucidation of molecular mechanisms of stationary phase mutation in *P. putida*. Our pioneering study of stationary phase mutagenesis in *P. putida* indicated that mutational processes in growing and starving *P. putida* must be different (Kasak *et al.*, 1997). Therefore, the main aim of my dissertation was to enlighten the molecular mechanisms underlying stress-induced

stationary phase mutation in *P. putida* with the special emphasis on some of the conventional factors controlling the mutation frequencies in bacteria under stressful conditions, namely, the involvement of DNA mismatch repair pathway and oxidative damage defense systems in avoidance of stationary phase mutations.

2.2. Changes in spectrum of mutations during starvation of *P. putida* (Reference I)

Previous results with *P. putida* test system based on the promoterless *pheBA* operon in plasmid pEST1414 indicated that base substitutions, deletions, insertions as well as transposition of chromosomal transposon Tn*4652* resulted in creation of functional promoter for activation of *pheBA* genes (Kasak *et al.*, 1997). In addition, the prevailing types of mutations in growing and stationary phase cells were different. However, in those studies the data of Phe^+ mutational spectrum of starving cells was not systematically obtained as the results of sequencing of Phe^+ mutant plasmids collected from Phe^+ mutants in different days of starvation were summarized. Those studies did not pay attention to possible effect of duration of starvation on Phe^+ mutational spectrum. Therefore, it was of interest to find out whether the time spent in starvation conditions could influence the genotypic changes of Phe^+ mutants of *P. putida*. For that purpose, we analyzed Phe^+ colonies emerging on phenol-minimal media during one-week period of starvation on each day separately. For the detection of Phe^+ mutants, *P. putida* wild type strain PaW85 carrying the promoterless *pheBA* operon in plasmid pEST1414 was starved on phenol minimal media and accumulation of Phe^+ mutants was observed (Fig. 1, ref. I). Phe^+ mutation spectra was obtained by analyzing ~250 bp region upstream of the *pheBA* operon in plasmid pEST1414 containing possible material for creation of promoters (Fig. 2, ref. I). Notably, approximately one-third of all stationary phase mutations were generated due to the insertion of transposon Tn*4652* into pEST1414 (Fig. 1, ref. I). These Phe^+ mutants containing insertions of Tn*4652* were excluded from our analysis.

Data of the Phe^+ mutation spectra indicated the characteristic spectra of mutations depending on the physiological state of bacteria (Table 2, ref. I). Importantly, there were significant changes in Phe^+ mutation spectrum during the time of starvation studied (Table 2, ref. I). One particular mutation type, the C-to-A transversion, was most dominating Phe^+ mutation type among starving cells on days 3 to 5. Interestingly, the proportion of C-to-A transversion declined among Phe^+ mutants that appeared on day 7. At the same time, the proportion of another base substitution mutation, G-to-T transversion, was found in almost equal proportions among Phe^+ mutations studied during the

period of starvation. When the mutation spectra of early-arising and late-arising Phe^+ mutants were compared then the significant differences in distribution of deletions depending on the duration of carbon source starvation was seen as well (Table 2, ref. I). The rare deletions ranging from 6 to 45 bp detected among the Phe^+ mutants that emerged on selective plates on days 3 to 5 were not found later. Instead, there was significant increase in the amount of small, 2- to 3-bp deletions among promoter-creating mutations during starvation-period studied, being most pronounced when spectrum of mutations obtained from Phe^+ mutants appeared on days 3 and 4 was compared to that of day 7. In agreement with previous results of Kasak *et al.* (1997), there was high proportion of 6 to 45 bp-range deletions in Phe^+ mutation spectra in addition to mutational hot spot C-to-A transversion observed in growing bacteria of *P. putida* PaW85 [Table 2, ref. I; (Kasak *et al.*, 1997)].

In summary, populations of stationary phase *P. putida* are remarkably dynamic, as according to our model system, already the 6-day-long period of starvation for carbon source creates significant variability in mutation types. In the beginning of starvation the promoter for the transcription of the *pheBA* operon is generated mostly due to C-to-A transversion in one potential –10 hexamer, whereas in later period of starvation this mutation type declines and frequency of 2- to 3-bp deletions increases. Therefore, the mutational processes in cells starving for a short time must be also different from those occurring during longer period of starvation. Owing to this, we were further interested to find out what is/are the molecular mechanism(s) responsible for the observed changes in mutational spectra in starving *P. putida* cells.

2.3. The mechanisms of stationary-phase mutations in *P. putida*

2.3.1. Deficiency of MutY leads to elevated frequency of C-to-A and G-to-T transversions (Reference I)

The specific spectrum of mutations is influenced by a variety of factors, including the efficiency of DNA replication and repair as well as lesions in DNA caused by different DNA-damaging agents. Under conditions of nutrient stress when DNA replication is minimal and more erroneous due to error-prone DNA replication, DNA damage might have an important role in generation of mutations. Therefore, it is reasonable to assume that DNA damage may be responsible for stationary phase mutation. However, in order to find out whether DNA damage could lead to stationary phase mutation it has to be clarified whether defects in DNA defense systems or repair pathways increase the rate of stationary phase mutation. In fact, there are several reports indicating the

insufficiency of DNA repair systems in starving *E. coli* (Foster, 1999a). From another part, it is demonstrated that oxidative DNA accumulation is also one of the (if not the major) contributors to the generation of stationary phase mutagenesis (Bridges, 1998). Reactive oxygen species that are generated as byproducts of oxygen metabolism interact with DNA bases to form lesions (see section 1.2). Among many types of oxidatively damaged bases, oxidized guanine (8-oxoG) is most common in cells and has gathered most interest concurrently with its recently discovered secondary oxidation products. One should also consider an oxidatively damaged adenine as a potentially mutagenic lesion (Kamiya, 2003). Altered DNA bases generated due to oxidation frequently change the base pairing ability. While guanine base normally pairs with cytosine only, 8-oxoG has the ability to pair in addition to cytosine also to adenine and failure to repair the A:8-oxoG mispair may lead to elevation of G:C-to-T:A transversions.

It is known that GO repair enzyme MutY (DNA adenine glycosylase) removes adenine from A:8-oxoG mispairs in double helix (Michaels *et al.*, 1992a). In case MutY enzyme fails to be operative on its substrate, the frequency of G:C-to-T:A transversions increases in *E. coli* cells (Michaels and Miller, 1992; Nghiem *et al.*, 1988). Furthermore, study with MutY-defective tryptophan auxotrophic *E. coli trpA23* strain indicated increased mutation frequency to Trp^+ due to G:C-to-T:A transversions under prolonged tryptophan starvation conditions (Bridges *et al.*, 1996; Bridges and Timms, 1997). So, drawing parallels, as Phe^+ phenotype in wild type *P. putida* cells was mostly generated as a result of C-to-A transversions we hypothesized that maybe, at least in the beginning of starvation period, there could be considerable amount of oxidative DNA damage responsible for the mutation spectra and the occurrence of this type of transversion may reflect the inefficiency of MutY in stationary phase bacteria.

In order to examine whether the *mutY* background could affect the nature of the spectrum and the frequency of appearance of Phe^+ mutants we created a *mutY*-defective *P. putida* strain and used the phenol starvation assay described previously (Kasak *et al.*, 1997). The *P. putida mutY*-deficient strain showed approximately 50-fold increase in promoter-creating Phe^+ mutation frequency when Phe^+ mutants of growing bacteria were compared to the wild type strain. Moreover, when the *P. putida mutY*-deficient strain was starved on phenol minimal media, the frequency of appearance of Phe^+ mutants was significantly higher in MutY-deficient strain compared to that of wild type strain during all 7 days studied (Table 4, ref. I). Thus, presumably MutY is involved in avoidance of mutations in both growing and stationary phase bacteria.

Still, the average number of Phe^+ colonies accumulating on phenol minimal media per day per 5×10^8 cells plated declined in case of *mutY*-defective strain (Table 4, ref. I). Importantly, as the lack of MutY caused mutator phenotype in *P. putida* and this could have increased the frequency of deleterious mutations

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in population, we studied the viability of MutY-defective strain on selective media. Compared to wild type, the viability of *mutY*-defective bacteria was not declined during the days of starvation on phenol minimal media (data not shown). Thus, the decline in accumulation rate of Phe^+ mutants was not caused by the higher mortality of *mutY*-defective cells, rather the presence of high number of Phe^+ mutants on selective media could inhibit further accumulation of mutants. To test this, we plated different amounts of *mutY*-deficient *P. putida* bacteria onto selective media and observed that large number of Phe^+ mutants indeed inhibited accumulation of mutants (Table 4, ref. I).

The analysis of mutation spectra of mutants collected from selective plates on day 2, 3 and 6 revealed that most of the Phe^+ mutants contained either C-to-A or G-to-T transversions with former represented more frequently (Table 3, ref. I). Both transversions created functional promoters for transcription of the *pheBA* operon in the same sites as we had characterized before (Fig. 2, ref. I). Remarkably, no other types of mutation were found although in starving bacteria some mutants contained changes we could not determine as occurred out of the sequencing area (Table 3, ref. I). Thus, these results demonstrated the role of MutY in avoidance of C-to-A transversions in both growing and starving cells of *P. putida* referring that efficiency of GO repair system in avoidance of mutations is important in starving bacteria.

2.3.2. Involvement of oxidative DNA damage defense systems in avoidance of stationary phase mutations in *P. putida*

One of the pathways dealing with the problem of oxidative DNA damage is oxidized guanine repair system. In order to prevent mutagenic effect of DNA replication errors caused by oxidized guanine, bacteria can utilize GO repair system that involves DNA glycosylases MutY and MutM, and MutT, the latter possessing preventive activity by eliminating mutagenic 8-oxodGTP from nucleotide pool (Michaels and Miller, 1992; Michaels *et al.*, 1992b). Inefficiency of MutT or MutY leads to strong mutator phenotype in *E. coli* whereas mutation frequency is not so much affected by MutM deficiency (see sections 1.3.2 and 1.7.2.3).

So far, the field of DNA repair and defense mechanisms against oxidative damage in *Pseudomonas* species is not extensively investigated. Among *P. aeruginosa* mutator strains isolated from CF patients lungs were found *mutY*-deficient strains but no alterations in *mutM* and *mutT* genes were determined (Oliver *et al.*, 2000; Oliver *et al.*, 2002b). Curiously, the functionality of GO repair system of *P. aeruginosa* was assessed only in *E. coli* (Oliver *et al.*, 2002b). The work done with *P. fluorescence* in searching efficient root-tip colonizers revealed the best mutant *P. fluorescence* strain of competitive colonizers to bear alteration also in *mutY* gene (de Weert *et al.*, 2004).

Our previous study indicated that *mutY* deficiency causes mutator phenotype also in *P. putida* (Ref. I). Considering that accumulation of oxidative DNA damage is characteristic to starving bacterial population and as the results of our study indicated the importance of one of the GO repair pathway enzymes (MutY) in avoidance of transversions both in growing and starving bacteria, the question of significance of activity of other enzymes of GO repair pathway, namely that of MutM and MutT, arised. We were also interested in whether oxidative DNA damage defense provided by Dps could be important in avoidance of stationary phase mutagenesis in *P. putida*. Further studies were dedicated to find the answer to these questions.

2.3.2.1. Efficiency of GO repair in avoidance of mutations (Reference II)

In order to further assess the role of GO repair system in avoidance of mutations in *P. putida* we constructed GO repair deficient strains PaWMutM and PaWMutT. According to the knowledge based on *E. coli*, repair deficient strains exhibit high spontaneous mutation frequencies (Miller, 1996). Therefore, we examined the base substitution mutation frequencies in growing populations of MutY-, MutT- and MutM-deficient *P. putida* strains by using chromosomal Rif^R system that enables to detect Rif^R colonies occurring due to single base changes in the *rpoB*-encoded β-subunit of RNA polymerase (Garibyan *et al.*, 2003; Jin and Gross, 1988). The total number of rifampin resistant mutants was counted on the next day after plating. Additionally, we used the test system which enabled the measurement of the base substitutions eliminating TAG stop codon introduced into the phenol monooxygenase encoding gene *pheA* locating in reporter plasmid pKTphe22TAG (Tegova *et al.*, 2004). In case of this test system we counted Phe^+ colonies appearing onto phenol-minimal plates on day 2 after plating. Similarly to the previous study (Ref. I), inactivation of MutY caused mutator phenotype in growing cells of *P. putida*. The median values of frequency of spontaneous Rif^R and Phe^+ mutations were increased ~ 90-fold and ~ 80-fold respectively, compared to wild type strain (Table 2, ref. II). However, the contribution of MutM and MutT to the avoidance of mutations in growing *P. putida* cells was modest. Only 2- to 3-fold increase in frequency of Rif^R mutation was seen in MutM- and MutT-deficient strains compared to wild type, and the Phe^+ mutation frequency was even smaller (Table 2, ref. II). For comparison, spontaneous mutagenesis was increased 20- to 100-fold in *mutY*-deficient (Nghiem *et al.*, 1988) and 10- to 14-fold in *mutM*-deficient *E. coli* cells (Cabrera *et al.*, 1988; Michaels *et al.*, 1992a), whereas lack of the MutT activity led to strong mutator phenotype as it increased the occurrence of transversions 1000-fold compared to wild type (Yanofsky *et al.*, 1966). Thus, according to the results, DNA adenine glycosylase MutY, but not other GO repair enzymes, is mainly involved in the prevention of base substitution mutations in growing cells of *P. putida*.

2.3.2.2. The antimutator effect of MutT enzyme depends on the growth phase of bacteria (Reference II)

We have previously shown that the lack of MutY enzyme activity causes elevated mutation frequency in starving *P. putida* (Ref. I). Therefore we asked, whether MutM and MutT proteins are involved in avoidance of oxidative damage-caused mutations in starving populations of *P. putida.* For that purpose we used previously described starvation assay which enables us to study different mutation types separately (e.g., various base substitutions and 1-bp deletions) in carbon starving *P. putida* cells (Tegova *et al.*, 2004). Thus, in order to monitor the emergence of Phe^+ revertants formed either due to frameshift or base substitution mutations, *P. putida* GO repair deficient and wild type cells carrying these test systems were plated onto phenol minimal plates and were let starved for a carbon source for two weeks. The results of starvation assay detecting the occurrence of frameshift mutations revealed that the frequency of accumulation of 1-bp deletants was not affected by the lack of GO repair proteins (Ref. II). However, the frequency of base substitution mutation was remarkably increased in PaWMutY (about 100-fold) and PaWMutT (about 75-fold) strains compared to that of wild type strain (Fig. 1A, ref. II). In contrast, the accumulation of Phe^+ revertants was not significantly affected by the *mutM*-deficiency when compared to the results of wild type strain (Fig. 1B, ref. II). Thus, MutY and MutT proteins are involved in lowering the base substitution mutations in starving *P. putida*. While the activity of *P. putida* MutY is essential in decreasing the occurrence of mutants through the growth cycle of bacteria, the effect of MutT is observed only in bacteria starving for the carbon source and not in growing cells, which hints to the involvement of some backup enzymes for MutT activity in growing bacteria (see below for discussion).

Much of the oxidation occurs in the nucleotide pool level and to minimize the incorporation of oxidized nucleotides into DNA, the clean-up mechanisms are evolved [see section 1.4 and 1.4.1; (Russo *et al.*, 2004)]. The known function of MutT is the cleansing of the nucleotide pool from 8-oxodGTP. So, when MutT is not functional, oxidized dGTP is readily inserted opposite dA and dC during replication and leads to A:T-to-C:G transversions (Maki and Sekiguchi, 1992; Tajiri *et al.*, 1995). According to the high frequency of mutations in starving *mutT*-defective *P. putida*, there must still be significant proportion of oxidized dGTP existing in the nucleotide pool of starving bacteria. Therefore, in order to clarify the question of functionality of GO repair enzyme MutT during starvation, we characterized the mutation spectra of the strain PaWMutT by analyzing Phe^+ mutants that appeared onto selective plates during 2-week starvation period due to base substitution in TAG stop codon within *pheA* gene. In case of *mutT*-defective *P. putida*, mostly A-to-C (70%) and T-to-G (20%) transversions were determined in starving bacteria (Table 3, ref. II). This result is consistent with the known and above stated functions of MutT in *E. coli*.

However, while the inactivation of *mutT* in *E. coli* leads to strong 1000-fold increase in spontaneous mutation frequency of A:T-to-C:G transversions (Yanofsky *et al.*, 1966), the lack of MutT activity affected the mutation frequency of growing bacteria only slightly in *P. putida* (Table 2, ref. II). What could be the reason of the low mutation frequency in growing *mutT* bacteria? MutT protein, firstly characterized in *E. coli* (Bhatnagar and Bessman, 1988), is a member of Nudix family of hydrolases. According to the genomic database of *P. putida*, there are 10 putative Nudix family proteins in this bacterium. For example, in *E. coli*, there are 13 Nudix hydrolases (McLennan, 2006). The proteins of Nudix family have various substrates among which there are intact and oxidized nucleotides as well as cellular metabolism endproducts (see section 1.4). Studies with *E. coli* have shown that at least three other proteins besides MutT are able to degrade 8-oxodGTP in growing cells although their antimutator effect is lower than that of MutT (see section 1.4.1). Considering this, one may assume that other Nudix proteins serve as backup enzymes for MutT in our study. However, the possible effects of these proteins in starving bacteria have not been studied so far. The field of Nudix hydrolases of *P. putida* is not investigated at all either. Nonetheless, we hypothesize that some of the other 9 Nudix family members of *P. putida* could function as backup enzyme of the MutT, possibly in a growth-phase dependent way. Thus, any of the Nudix hydrolase family proteins expressed in growing bacteria could suppress the mutator effect of *mutT* in growing *P. putida*. As oxidized guanine accumulates also in starving bacteria, *mutT* role may be more pronounced in cleansing the nucleotide pool in stationary phase bacteria. Also, drawing parallels with *P. aeruginosa*, there can be found 9 putative Nudix family proteins in its genome. Thus, similarly, any of these proteins in *P. aeruginosa* could function as a backup enzyme for MutT, since among mutator strains isolated from CF patients lungs, no *mutT* mutators were found either (Oliver *et al.*, 2000; Oliver *et al.*, 2002b).

2.3.2.3. Functions of MutY and MutM glycosylases in avoidance of stationary phase mutation (Reference II)

In order to further address the question of functionality of GO repair enzymes MutY and MutM in avoidance of mutations that are presumably caused as a result of 8-oxoG in DNA we analyzed the Phe^+ mutation spectra in *mutY*- and *mutM*-deficient strains and wild type strain.

The characterization of the Phe^+ mutation spectra revealed that the prevailing type of mutations in wild type bacteria was T-to-C (77%) transversion and the other types of mutations like T-to-G or A-to-G were represented in modest proportions (Table 3, ref. II). Admittedly, the mutation spectra of wild type strain were different than in GO repair deficient strains. If GO repair were not functional in starving wild type bacteria then there would not have been any difference in mutation spectra between starving wild type and GO-defective

populations. Therefore, according to the distinct mutation spectra observed in GO repair deficient and wild type strain we conclude that GO repair system is functional during starvation period studied in *P. putida*. Analysis of the Phe^+ mutation spectra of MutY-defective strain revealed as anticipated, predominantly G-to-T transversions (85%) with lesser amount of T-to-C transversions (12%) (Table 3, ref. II). The deficiency in MutY enzyme was responsible for high occurrence of G-to-T transversions also in our previous study (Table 3, ref. I). Notably, G-to-T transversions led to 20-fold increase in tryptophan prototrophs in *mutY*-defective *E. coli* (Bridges *et al.*, 1996). Thus, the function of MutY enzyme in *P. putida* seems to be similar to the role of MutY in the model organism *E. coli*.

Differently from other GO repair system enzymes MutY and MutT, the absence of MutM did not elevate the Phe^+ mutation frequency in starving *P. putida*. The same negligible effect of MutM deficiency to stationary phase mutations has been shown also in *E. coli* (Bridges *et al.,* 1996). Nevertheless, the proportion of G-to-T transversions was significantly increased in PaWMutM strain compared to wild type strain (Table 3, ref. II). The difference of mutation spectra refers to functionality of MutM in starving *P. putida*. However, as the mutation frequency did not increase in PaWMutM strain, it hints that the backup enzymes of MutM are in abundance and therefore the level of oxidized guanine or its secondary oxidation products may be low. It is known that in *E. coli*, there are other DNA glycosylases/AP lyases able to remove also 8-oxoG from different mispairs and thereby serve as a backup enzymes for MutM (see section 1.3.2.3). Specifically, both Nei (endonuclease VIII) and Nth (endonuclease III) can eliminate 8-oxoG and double mutant defective in Nei and Nth activity showed strong mutator phenotype (Jiang *et al.*, 1997a). The genomic database of *P. putida* does not indicate the presence of Nei homologue in this organism. However, Nth homologue exists in genome of *P. putida* and its deduced amino acid sequence is 67.8% identical to Nth protein sequence of *E. coli*. Therefore, we hypothesize that Nth protein activity may suppress the otherwise expected high mutation frequency of MutM-defective *P. putida*.

Although the activity of backup enzymes of MutM seems to be the most reasonable explanation of the low mutation frequency in *P. putida mutM*-defective strain, some other possibilities could also be considered. Specifically, it has been shown, that MMR contributes to the avoidance of mutations caused possibly due to 8-oxoG. Namely, overexpression of MutS significantly reduced the rate of G:C-to-T:A transversions in both starving and growing *mutM*-defective *E. coli* strain suggesting its ability to correct A:8-oxoG mispairs (Zhao and Winkler, 2000). Also, human homologs of MutS have been shown to bind to mispairs containing 8-oxoG (Mazurek *et al.*, 2002) and furthermore, overexpression of MutM in a *mutH*-defective *E. coli* strain reduced the rate of G:C-to-T:A transversions (Wyrzykowski and Volkert, 2003). Thus, one may hypothesize that MMR may be one of the backup mechanisms for prevention of

the harmful effects of 8-oxoG in *P. putida* and interplay between MMR and BER pathways may occur in this organism as well. From another point, genetic analysis of *E. coli* has indicated that 8-oxoG is also subject of transcription-coupled repair (TCR) despite its efficient bypass by RNA polymerase (Bregeon *et al.*, 2003). Overall, the conducted studies suggest that there is also a competition between BER and TCR for the repair of 8-oxoG and additional proteins, possibly the components of NER and MMR, could participate in the process as well.

In conclusion, our results indicate that the activity of GO repair enzymes MutT and MutY with a lesser contribution of MutM, significantly reduce the stationary phase mutation rates in *P. putida*. Although, the involvment of MutY must be the dominant mechanism dealing with 8-oxoG produced in DNA, the possible contribution of other DNA repair enzymes and backup systems shows the significant threat the oxidative damage poses to starving bacterial population.

2.3.2.4. Involvement of secondary oxidation products in stationary phase mutagenesis in *P. putida* (Reference II)

Several different studies have indicated that oxidative stress yields to mutation spectra with G:C-to-T:A and G:C-to-C:G transversions (Neeley and Essigmann, 2006). For example, in starving population of *mutY*-deficient *E. coli* there were in addition to expected G:C-to-T:A transversions also G:C-to-C:G base substitutions (Zhang *et al.*, 1998). While the occurrence of G-to-T transversions can be explained by the presence of 8-oxoG, G-to-C mutation indicated the involvement of some other oxidized nucleotide. Quite recent *in vitro* studies have uncovered the feature of guanine and 8-oxoG to be susceptible for further oxidation and that secondary oxidation products constitute already a significant block to replication (Neeley and Essigmann, 2006). Thus, it means that when a guanine secondary oxidation product is located in DNA, it stalls the DNA polymerase III and translesion DNA synthesis polymerases might be recruited in error-prone DNA replication past the replication-blocking lesions (Nohmi, 2006). Both error-prone DNA polymerases Pol IV and Pol V contribute to the stationary phase mutagenesis (Bhamre *et al.*, 2001; Nohmi, 2006). However, Pol V has more pronounced role in mechanism of guanine oxidative mutagenesis in *E. coli* than Pol IV or Pol II (Neeley *et al.*, 2007). In fact, the requirement of Pol V to generate G-to-C transversions has been shown also in starving *E. coli mutY* population (Timms *et al.*, 1999). Although in the chromosome of *P. putida* there cannot be found genes to encode Pol V, the Pol V homologue RulAB is encoded by the conjugative TOL plasmid pWW0 (Greated *et al.*, 2002). Our research group has shown that RulAB increases evolutionary fitness of *P. putida* by allowing the emergence of advantageous mutations during starvation (Tark *et al.*, 2005). Therefore, taking into account the recent exciting discovery of secondary oxidation products of guanine and that Pol V

participates in that process in *E. coli*, we decided to use *rulAB*-encoded Pol V homologue RulAB in order to find out whether secondary oxidation of guanine could be involved in stationary phase mutation in *P. putida*.

To study the possible linkage between oxidative damage of DNA and RulAB-dependent mutagenesis in stationary phase mutation in *P. putida*, we used the assay system measuring the reversion of TAG stop codon in the *pheA* gene. We found that the presence of the *rulAB* genes in the chromosome of the wild type and GO repair deficient strains did not significantly affect the frequency of accumulation of Phe^+ revertants compared to the strains lacking *rulAB* genes (Fig. 2, ref. II). Additionally, we analyzed the mutation spectra of the Phe^+ revertants picked up from phenol minimal plates during 2-week starvation. When we compared the sequencing data of Phe^+ mutants gathered from the strains bearing *rulAB* genes in the chromosome to the results of those strains without *rulAB* genes in the chromosome, we found differences between mutation spectra of base substitution mutations in case of wild type and PaWMutM strain (Table 3, ref. II). This result indicates that RulAB (Pol V) is involved in generation of base substitution mutations in stationary phase *P. putida*.

Surprisingly, the presence of RulAB did not reveal the expected G-to-C base substitutions. So, one could hypothesize that even when secondary oxidation of guanine or 8-oxoG does occur, it may remain to too low level as in the opposite situation we could detect G substitution mutations. Notably, hydantoin lesions produced from 8-oxoG are substrates to MutM and Nei in *E. coli* (Hazra *et al.*, 2001b; Leipold *et al.*, 2000). Thus, it is tempting to speculate that some of the DNA glycosylases may recognize and eliminate secondary oxidation products of guanine from DNA in *P. putida*. Therefore, it may also be the reason why we do not see the effect of secondary oxidation products of guanine with our test system.

Despite the presence of *rulAB* genes in the chromosome of *P. putida* strains, the proportion of G-to-C mutations in Phe^+ mutation spectra did not increase, instead, our analysis revealed an enhancement of unexpected base substitution type, namely the proportion of A-to-G and A-to-C substitutions was increased (Table 3, ref. II). Particularly, in case of wild type strain PaW85, the proportion of A-to-G transitions was increased from 6% to 27% when *rulAB* genes were introduced into its chromosome. The proportion of A-to-C base substitution was also significantly enhanced (from 0 to 2%) in wild type strain carrying the Pol V homologue in the chromosome whereas this type of mutation was not detected in mutation spectra of wild type PaW85 without *rulAB* genes in the chromosome. At the same time, as the proportion of A-to-C transversions was not elevated in MutT-defective *P. putida* strain carrying *rulAB* genes in the chromosome compared to MutT-defective strain without *rulAB* genes (Table 3, ref. II), it means that this type of mutation is not generated by insertion of 8-oxodGTP from the nucleotide pool but by other mechanisms. According to

the literature, there is evidence that despite of the lower abundance than that of 8-oxoG, oxidized adenine has also significant mutagenic potential in cell as leads to replication fork block that is processed by mutagenic replication by error-prone DNA polymerases (Frelon *et al.*, 2002; Kamiya, 2003). The bypass of 2-OH-Ade in DNA by archaeal error-prone DNA polymerase as well as by Klenow fragment in *E. coli* has been shown to lead to A-to-G, A-to-C and A-to-T base substitutions (Barone *et al.*, 2007; Kamiya and Kasai, 1997; Nohmi, 2006). Therefore, as Pol V is also one of the error-prone DNA polymerases and is able to synthesise past the damaged nucleotides distorting DNA helix then one may speculate that the effects of *P. putida* Pol V homologue RulAB we found are due to the presence of oxidatively damaged adenine in DNA.

In conclusion, at least in our test system, the guanine secondary oxidation does not remarkably affect the frequency of accumulation of Phe^+ mutants in the presence of DNA polymerase Pol V homologue RulAB compared to the strains not bearing *rulAB* genes in the chromosome. Nevertheless, significant enhancement of adenine substitutions in the presence of RulAB indicates that oxidation product(s) of adenine constitute one of the important sources of stationary phase mutations in *P. putida*.

2.3.2.5. The effect of Dps on stationary phase mutation in *P. putida* (Reference II)

So far, we have considered the role of base excision repair of endogenously generated oxidative damage by oxidized guanine repair system enzymes in stationary phase mutagenesis in *P. putida*. However, it is known that DNA can be protected from exogenous oxidative damage by stationary-phase-specific DNA-binding protein Dps (Minsky *et al.*, 2002), which is highly abundant in *E. coli* cells being under conditions of nutrient or oxidative stress (Ali Azam *et al.*, 1999). The protection is promoted by the formation of DNA-Dps cocrystals, and it is proposed that DNA protection in starved bacteria that involves Dps protein could be a general mechanism employed during starvation (Frenkiel-Krispin *et al.*, 2001). In fact, the role of Dps in mutagenesis has been investigated in the presence of hydrogen peroxide and in those conditions the Dps-defective *E. coli* strain revealed about 10-fold increase in G:C-to-T:A transversions (Martinez and Kolter, 1997). However, whether the Dps-dependent DNA defense mechanism could be applicable to stationary phase mutagenesis under physiological conditions was not investigated in these studies.

Dps homologs have been found in many distantly related bacteria and *P. putida* is not an exception here (www.tigr.org). In order to establish the role of *P. putida* Dps in protection against oxidative damage we constructed a *dps*-defective strain of *P. putida* and performed the oxidative-stress assay [similar to that conducted in *E. coli* (Nair and Finkel, 2004)] with wild type and *dps* mutant strain. We found that *P. putida* Dps-defective strain PaWDps was more sensitive to the added 200 mM hydrogen peroxide than the wild type strain (Fig. 3,

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ref. II). The viability of PaWDps strain decreased rapidly (about 10 000-fold) already after 30 minutes of H_2O_2-treatment whereas the viability of wild type strain was reduced 300-fold. During the period of the experiment bacteria from both strains adapted somewhat to hydrogen peroxide stress but the maintained viability was still lower in PaWDps strain. Therefore, Dps is involved in defending *P. putida* against exposure of hydrogen peroxide.

As a next step, we followed the route of our previous experiments. In order to find out whether Dps activity could contribute to mutational processes in stationary phase *P. putida*, we performed the starvation assay by measuring the frequency of accumulation of Phe^+ mutants in wild type and PaWDps strain generated due to reversion of TAG stop codon locating in the *pheA* gene within the test plasmid. We expected to see the effect of Dps specifically after prolonged starvation of *P. putida* as the studies with *E. coli* have been shown that the amount of Dps is increased in cells being in stationary phase for 3 days. However, somewhat surprisingly we did not see the difference in Phe^+ mutation frequencies between wild type and PaWDps strain (Fig. 4A, ref. II). The minor difference in accumulation curves of Phe^+ mutants was not statistically significant. Additionally, the mutation rate was not increased compared to *mutY*-defective strain in case when both *dps* and *mutY* were non-functional (Ref. II). These unexpected results led us to suspect that Dps may not be involved in avoidance of stationary phase mutation in *P. putida*. In the hope to see some difference in DNA level we also analyzed the sequence of Phe^+ mutants picked up from phenol minimal medium during the 10-day starvation period. However, the spectra of mutations in PaWDps strain resembled that of wild type bacteria (Fig. 4B, ref. II). Owing to this, we conclude that Dps has no role in avoidance of stationary phase mutations in *P. putida*.

Given that Dps protein is abundant in stationary phase bacteria and the DNA is packed into DNA-Dps co-crystals then it is amazing how the DNA repair proteins are recruited to the damaged site and perform their job in highly condensed DNA structure. However, as DNA in eukaryotes is also packed into nucleosome structures then the situation must be similar. *In vitro* study of repair of DNA lesions in reconstituted eukaryotic chromatinized DNA showed that nucleosomal DNA repair is slower than that of naked DNA (Hara *et al.*, 2000). Considering this, when Dps protein is absent in stationary phase bacteria then DNA must be more easily accessible to repair proteins and repair must occur rapidly or possibly at a rate similar to growing bacteria where there is no Dps. It could explain the comparable mutation frequency of Dps mutant strain and wild type strain. On the other side, oxidative DNA damage is important source of mutations in starving bacteria and Dps has also the ability to sequester Fe-ions, a component of Fenton reaction producing harmful hydroxyl radicals. Therefore, in the absence of Dps, DNA must be more vulnerable to the effects of active oxidative species and presumably, mutation frequency should be high. Possibly, the endogenously formed oxidative species and the DNA damage they

generate are efficiently eliminated. Given the high abundance of enzymatical systems and multiple DNA glycosylases dealing with elimination of damaged bases as well as contribution of DNA repair pathways it may not be surprising at all.

In summary, although it is evident that oxidative damage to DNA plays a substantial role in stationary phase mutagenesis in *P. putida*, Dps protein seems not to be essential in avoidance of mutations in starving bacteria. Yet, the protection against exogenous DNA-damaging agents by Dps is still conferred.

2.3.3. Lack of stationary phase sigma factor of RNA polymerase affects the spectrum of stationary phase mutation in starving *P. putida* (Reference I)

Our study of stationary phase mutagenesis with the test system employing the promoterless *pheBA* operon as a reporter in plasmid pEST1414 showed that the spectrum of stationary phase mutations among early-arising mutants (collected on days 3 and 4) differed from that of later-arising ones (collected on days 6 and 7) in *P. putida* (Table 2, ref. I). We saw increase in occurrence of small deletions (e.g., 2- to 3-bp deletions) among promoter-creating mutations in late stationary phase Phe^+ mutation spectra of *P. putida* wild type strain PaW85[pEST1414]. This finding refers to some changes in the regulation of mutagenic pathways during starvation conditions. Studies with *E. coli* have shown that stationary phase sigma factor σ^S (RpoS) regulates the expression of genes involved in responses to diverse stresses including stationary phase, starvation, osmotic, acid, heat, and oxidative stress (Hengge-Aronis, 2002; Loewen and Hengge-Aronis, 1994). RpoS is shown to be a global transcriptional regulator also in *Pseudomonas* [reviewed in (Venturi, 2003)]. Thus, we asked whether RpoS could be involved in generation of changes we saw in stationary phase Phe^+ mutation spectra in *P. putida*. In order to test that, we used *P. putida rpoS*-defective mutant strain PKS54 that is a derivative of *P. putida* wild-type strain PaW85 (Ojangu *et al.*, 2000). Both wild type and PKS54 strains carrying the test system described above were starved on phenol-minimal media. The frequency of accumulation of Phe^+ mutants was lower in PKS54 strain compared to the wild type strain during the first five days of starvation whereas on days 6 to 7 the mutation frequency of PKS54 strain was increased compared to the wild type strain (Fig. 1, ref. I). However, the lower mutation frequency of PKS54 seen during the first 5 days of starvation cannot be the result of decreased viability as the survival of PKS54 strain was comparable to wild type strain (Ilves *et al.*, 2001). Surprisingly, when we analyzed the Phe^+ mutants of starving PKS54 strain, then it came out that the higher rate of accumulation of Phe^+ mutants was caused mainly by activation of transposition of IS*1411* (Fig. 1, ref. I). The mobile element IS*1411* locates downstream of the *pheBA* genes in

plasmid pEST1414 from where it can insert upstream of the promoterless *pheBA* operon and activate these genes by outward-directed promoters at its left end (Kallastu *et al.*, 1998). Hence, the activation of the transposition of IS*1411* seems to be negatively controlled by RpoS, suggesting that some RpoS-dependent factor is needed to downregulate IS*1411* transposition activity. As a next step, we characterized those Phe^+ mutants that emerged on phenol-minimal media on day 4 and days 6 to 7 and were not caused by insertion of IS element. The sequencing of Phe^+ mutant plasmids revealed that small deletions (2- to 3-bp) as well as small, 2- to 3-bp insertions were absent in RpoS-deficient *P. putida* (Table 5, ref. I). Thus, these results imply that some mutagenic pathway that leads to creation of small deletions and insertions in starving *P. putida* is positively regulated by RpoS. At the same time this effect of RpoS is more pronounced in those bacteria that have already starved for some days.

Still, why the proportion of microdeletions among promoter-creating mutations arises during later days of starvation? One of the mechanisms of creation of deletions presumes the formation of stem-loop structure in DNA strand (Balbinder *et al.*, 1993), but upstream of the *pheB* gene there cannot be found regions that could possibly form loops. The generation of deletions was elevated also by the mutator activity of DNA Pol I mutant variant (*polA1*) (Jankovic *et al.*, 1990). In that case the Pol I-induced deletions were associated with a 5´-GTGG-3´ sequence, which is a pause site for this protein and for other polymerases; however such region is missing upstream of the *pheB* gene. Bridges and Timms (1997) demonstrated that when *mutY trpA E. coli* cells were under trypthophan starvation then among Trp^+ phenotype-conferring mutations also small in-frame deletions were discovered (Bridges and Timms, 1997). However, the mechanism of the generation of microdeletions remained unknown.

Another possible mechanism to generate genetic diversity and therefore elevate the chance of beneficial mutation in stressed bacteria is the induction of error-prone DNA polymerases and there are several reports about their involvement in stationary phase mutation (see section 1.7.2.2). In case of *E. coli* FC40 model system, DNA Pol IV is required specifically for 1-bp deletions generating Lac^+ revertants in stationary phase bacteria (McKenzie *et al.*, 2001) and the cellular amount of Pol IV in *E. coli* FC40 is controlled by RpoS (Layton and Foster, 2003). The study of the involvement of error-prone DNA polymerase IV in stationary phase mutagenesis in *P. putida* indicated that this polymerase is specifically involved in generation of 1-bp deletions in populations that had starved for 2 weeks on phenol-minimal media (Tegova *et al.*, 2004). The experiments in our laboratory have not demonstrated the involvement of Pol IV in the occurrence of 2- to 3-bp deletions or insertions (unpublished data). Yet, the cells are able to bypass the majority of lesions in DNA by using either one or several error-prone DNA polymerases in concert (Friedberg, 2005; Rattray and Strathern, 2003). In fact, recent model of damage bypass synthesis demonstrated that replication fork did not stall at damage site

but proceeded, leaving a gap opposite the lesion for subsequent sealing by error-prone DNA polymerases and the number of gaps was increased in strains lacking error-prone DNA polymerases (Heller and Marians, 2006; Lopes *et al.*, 2006). Thus, one could hypothesize that some yet undiscovered interactions of translesion-synthesis polymerases may affect the mutation spectra in starving *P. vputida* and the occurrence of small deletions as well.

From another point, the occurrence of mutations may be also regarded as a consequence of ineffective DNA repair system. As we have ruled out that GO repair could be ineffective in avoidance of mutations in starving *P. putida*, maybe the inefficiency of some other DNA repair system could be responsible for the elevated level of mutations in stationary phase *P. putida*?

2.3.4. Contribution of DNA mismatch repair to stationary phase mutagenesis in *P. putida*

It was already in 1988 when F. W. Stahl proposed that stationary phase (adaptive) mutation may occur due to a decline in mismatch repair (MMR) (Stahl, 1988). Subsequent studies have shown indeed that one of the major mutation-prevention pathway, MMR might be downregulated in stationary phase (see section 1.7.2.1). Also, mutational spectrum of stationary phase mutation in FC40, the 1-bp frameshifts, suggested the involvement of a mismatch repair deficiency or insufficiency as small 1–4 nt insertion/deletions are substrates of MMR (see section 1.7.2.1). It has also been actively disputed about the temporal insufficiency of MMR during stationary phase mutation (see section 1.7.2.1). The idea was initially supported by Longerich *et al.* (1995) who demonstrated that when MMR is defective then the Lac^+ stationary phase mutation spectrum can be obtained in growing cells (Longerich *et al.*, 1995). It cannot be excluded also that cells exhibiting stationary phase mutation may have accumulated DNA lesions possibly created by error-prone DNA polymerase(s) and this may saturate the repair capacity of MMR proteins (Schaaper and Radman, 1989; Wagner and Nohmi, 2000). Therefore, we were interested to test the possibility that the mechanism of stationary phase mutation in long-term-starved *P. putida* population could be ascribed to malfunctioning of MMR in those cells.

2.3.4.1. Absence of MMR confers mutator phenotype in *P. putida* (Reference III)

In order to study the involvement of MMR in stationary phase mutagenesis in *P. putida*, we constructed *P. putida* MMR-knockout strains PaWMutS and PaWMutL. Considering that the deficiency in MMR enzymes results in mutator phenotype (see section 1.6.2) we first tested the ability of these strains to elevate

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spontaneous mutagenesis. To analyze this, we compared the base substitution mutation frequencies in growing populations of MutS- and MutL-deficient *P. putida* strains with that of wild type strain PaW85. We used chromosomal Rif^R system in case of which we plated bacteria onto rifampin plates and counted colonies after 24 hours. The other test systems based on measurement of base substitutions eliminating TAG, TGA or TAA stop codon in the phenol monooxygenase-encoding gene *pheA* located in reporter plasmids pKTphe22TAG, pKTphe22TGA or pKTphe22TAA, respectively (Tegova *et al.*, 2004). The results revealed that the mutator phenotype was caused in the absence of either MutS or MutL. The spontaneous frequency of mutation to rifampin resistance was about 1000-fold higher both in PaWMutS and PaWMutL strains compared to the wild type strain (Ref. III). The median values of the frequency of spontaneous Phe^+ mutations was increased about 50-fold in MMR defective strains compared to wild type strain (data not shown). Therefore, MMR is important in avoidance of mutations in growing bacteria.

2.3.4.2. MMR is functional during long-term starvation period of *P. putida* (Reference III)

As we saw significant increase in spontaneous base substitution mutation frequencies in growing MMR-defective populations, we asked whether MutS and MutL proteins could be involved in avoidance of mispair-caused mutations in starving populations of *P. putida*. For that purpose we used previously described starvation assay which enables us to study various base substitutions in carbon starving *P. putida* cells (Tegova *et al.*, 2004). Thus, in order to monitor the emergence of Phe^+ revertants formed due to base substitution mutations we plated MMR-deficient and wild type cells carrying tester plasmid containing either stop codon TGA, TAA or TAG introduced into the *pheA* gene, onto phenol minimal medium and compared the frequency of occurrence of Phe^+ mutants in MMR-deficient strains to that of wild-type strain PaW85.

The frequency of accumulation of Phe^+ revertants was approximately 25 times elevated in starved MMR-defective PaWMutS and PaWMutL strains compared to the wild type strain throughout all the starvation period studied (Fig. 1B, ref. III). Importantly, Phe^+ mutants appeared onto selective media with constant rate throughout the 2-week-period studied both in wild type as well as in MMR-defective strains (Fig. 1B, ref. III). The accumulation of the Phe^+ revertants in starving populations of *P. putida* wild type and MMR-defective strains measured in either TAA, TAG or TGA test systems was similar (Ref. III). Thus, these results indicate that firstly, MMR is involved in avoidance of base substitution mutations in starving *P. putida* cells and even more interestingly, the correction of mismatches is maintained throughout the starvation by MMR in *P. putida*. Furthermore, the increase of Phe^+ mutation frequency in MMR-deficient strains both in growing and starving conditions indicates that DNA synthesis goes on with considerable extent in starvation conditions and

the ability of MMR to correct mispairs is not diminished during starvation. Therefore, our results support the idea that even if the activity of MMR proteins really declines during the time of starvation its must remain functional for the DNA replication that takes place in those conditions. Foster (Foster, 1999b) and Bridges (Bridges, 1996) have earlier arrived to similar conclusion in *E.coli* studies and Bridges (1996) have implied that under the conditions of starvation there must be occurring considerably more DNA synthesis. The possibility that cells may utilize breakdown products of DNA or RNA, thus representing the phenomenon of DNA turnover, was proposed later (Bridges and Ereira, 1998).

According to our previous studies, the frequency of accumulation of 1-bp deletions was increased in wild type *P. putida* cells starved more than a week (Tegova *et al.*, 2004). Thus, we wanted to figure out whether this could be explained by malfunctioning of MMR during prolonged starvation. We used the assay system specifically measuring the reversion of +1 frameshift in the *pheA* gene. The results showed that frequency of accumulation of 1-bp deletants increased in time of starvation in wild type as well as in MMR-knockout strains (Fig. 1C, ref. III). Specifically, comparing the results of accumulation of Phe^+ mutants of the one-week period of starvation to that of longer-period of starvation (days 11–15) then the frequency of appearance of Phe^+ revertants was increased 10-fold in case of PaWMutS and 15-fold in strain PaWMutL. However, the above-presented results in Fig. 1C, ref. III show that the lack of MMR has less influence on the emergence of 1-bp deletants compared to that of Phe^+ revertants generated due to base substitutions (compare Fig. 1B and C, ref. III). It is possible that diverse types of mutations are corrected by MMR by different efficiency in starving *P. putida*. At the same time, as the frequency of 1-bp deletion mutations was elevated both in wild type and in MMR-deficient strains, it implies to the possibility that the increased accumulation rate of 1-bp revertants in long-term-starved *P. putida* wild type might be due to the increased level or activity of Pol IV. In fact, there is evidence that MMR may be insufficient due to its saturation with excess of DNA replication errors (Schaaper and Radman, 1989), and errors can arise also as a result of overproduction of Pol IV in *E. coli* (Wagner and Nohmi, 2000). So, taking into account the above-presented results and the literature data, the emergence of Phe^+ 1-bp revertants onto phenol-minimal media in long-term-starved *P. putida* wild type strain may in some extent be facilitated by partial titration of MMR as a consequence of increase of Pol IV-dependent errors in DNA during the time of starvation.

2.3.4.3. Spectra of stationary phase mutations are different in wild type strain and its MMR-defective derivatives (Reference III)

Our starvation assay based on detection of base substitutions indicated that MMR might be functional during the nutritional stress conditions. Owing to this, we were further interested to get more straightforward evidence of functionality of MMR during the time of starvation. In case the MMR activity really declines in stationary phase as proposed elsewhere (Harris *et al.*, 1999) in wild type cells then one should see the characteristic stationary phase mutation spectra which should be similar to that obtained in mutants that were picked up among MMR-defective *P. putida* population. Therefore, we asked whether the activity of MMR enzymes MutS and MutL could influence the spectrum of base substitution mutations and also, whether it is affected by growth phase of bacteria. For this purpose, we analyzed the DNA sequence of Phe^+ mutants collected either from growing or starving populations of wild type and MMR-defective strains carrying assay systems measuring the elimination of TAG, TAA or TGA stop codon in the *pheA* gene.

Firstly, we found differences between Phe^+ mutation spectra of growing and carbon-starving populations of wild type strain thus referring to the different mutational pathways depending on growth phase (Table 2, ref. III). Secondly, the spectrum of mutations depended on the sequence of the particular stop codon in the test system. Notably, T-to-C substitution was the most prominent type of base substitution, independent of growth phase and test system. The most heterogenous mutation spectrum in starving wild type population was seen in case of assay system that measured the elimination of TAG stop codon. Additionally, when we compared the spectra of base substitutions identified in starving populations of the wild type strain with that obtained in starving populations of MMR-defective strains then those were distinct too (Table 2, ref. III). If MMR were not functional in starving wild type bacteria then there would not have been difference in mutation spectra between starving wild type and MMR-defective populations. Therefore, this result supports our conclusion that MMR is functional at least during the 2-week starvation-period studied.

When looking the data obtained from analysis of Phe^+ mutation spectra one can see that in comparison to the wild type strain the spectrum of base substitutions obtained from revertants of MMR-defective strains was more homogenous, containing mostly two types of transitions – T-to-C and A-to-G (Table 2, ref. III). This result shows that similarly to MMR in *E. coli* (see section 1.6.2) the mismatch repair in *P. putida* is preferably involved in avoidance of transition mutations.

Additionally, the mutation spectrum of starving MMR-defective bacteria depended on which component of MMR was non-functional. This finding was most pronounced when the MMR-defective Phe^+ mutants carrying the assay system for eliminating TAG stop codon in the *pheA* gene were analyzed (Table 2, ref. III). The proportion of one particular A-to-G base substitution was signi-

ficantly increased among Phe^+ mutants of starving PaWMutS strain (70%) compared to mutants of growing cells (18%) whereas in case of PaWMutL this change was smaller (14% A-to-G mutations in growing bacteria versus 33% in starving cells). Thus, what could explain this finding? It is possible that the lack of either MutS or MutL may have different effect on DNA replication as according to the literature, both *E. coli* MutS and MutL are among the several DNA metabolism proteins that bind to the β processivity clamp of the replicative DNA polymerase III (see section 1.6.1.5) and through this may influence DNA repair and DNA replication. Moreover, as the clamp loader protein, MutS, MutL and all polymerases in *E. coli* bind on the same site to β clamp, it suggests that competitive interactions must take place upon binding to β sliding clamp (Lopez de Saro *et al.*, 2006). Indeed, it has been shown that processivity clamps regulate the exchange of replicative and repair DNA polymerases during DNA replication to overcome possible damage in DNA (Friedberg *et al.*, 2005; Indiani *et al.*, 2005). Admittedly, the specific peptide motif for interaction with β clamp that is found in most sequenced eubacterial MutS and MutL proteins (Dalrymple *et al.*, 2001) is also present in *P. putida* MutS and MutL protein sequence. Therefore, it can be hypothesized that *P. putida* MutS and MutL interaction with β clamp may influence the balance of competitive interactions between processivity clamp and different DNA polymerases, which in turn may result in different mutation spectra. While in case of *E. coli*, the contribution of error-prone DNA polymerase Pol V in base substitution mutations has been shown (Bhamre *et al.*, 2001), *P. putida* like many other bacterial species do not possess chromosomally encoded Pol V, which excludes the possibility that Pol V or its homologue is a competitor for the interaction with β clamp. However, despite the lack of the chromosomally encoded Pol V from genomes, these bacterial species carry the so-called "mutagenesis cassette" that may replace the functions of Pol V (Erill *et al.*, 2006; Galhardo *et al.*, 2005). Indeed, a multiple gene cassette – *lexA2-imuA-imuB-dnaE2* – was recently described in *Pseudomonas putida* (Abella *et al.*, 2004). In this operon, under the control of a second copy of the repressor protein LexA2 there is a protein exhibiting similarity to Y-family DNA polymerases (ImuB), and a second copy of an alpha subunit of the DNA polymerase Pol III (DnaE2). Thus, one could hypothesize that different interactions of these proteins with β clamp could contribute to the mutation spectra as well. In fact, our recent pioneering study of the involvement of ImuB and DnaE2 in stationary phase mutagenesis in *P. putida* discussed their possible role in affecting the polymerase traffic (Koorits *et al.*, 2007).

Differences in Phe^+ mutation spectra obtained from MutS- and MutL-defective strains can be explained also by the fact that although both MutS and MutL have been shown to interact with β sliding clamp, they are still involved in different steps of MMR, yet co-ordinately. As noted in the section 1.6.1.5 of literature overview, one possible role of interaction of MutS with β clamp may

be to help orient MutS on DNA providing the information of which strand is newly replicated. Latter may well account for MMR in *Pseudomonas* because differently from *E. coli*, genomes of *P. putida* as well as those of *P. aeruginosa* and *P. stutzeri* all lack DNA adenine methylase gene *dam* and therefore it is likely that no methylation occurs in their GATC sequences (Meier and Wackernagel, 2005). Thus, failure of strand discrimination signalling in case of nonoperative MutS protein in *P. putida* may affect the mutation spectra seen in starvation conditions.

Furthermore, pseudomonads also lack the *mutH* gene and it is not known which protein could provide the nick into nascent DNA strand. Intriguingly, it was recently reported about the finding of an intrinsic endonuclease activity of human MutLα (Kadyrov *et al.*, 2006). The endonucleolytic activity was guaranteed by the protein motif found in the C-terminus of human MutLα protein and in many bacterial MutL proteins except in *E. coli* MutL (Kadyrov *et al.*, 2006; Yang, 2007). Given that the same protein motif is present also in MutL protein of *P. putida* and *P. aeruginosa* (http://www.ncbi.nlm.nih.gov/blast) and that human MutLα endonuclease activity was stimulated by the MMR cofactors one can hypothesize that in case of pseudomonads, the MutS is oriented by β clamp and loaded onto DNA with strand bias and then MutL protein is induced for endonucleolytic activity.

Additionally, according to the study of Indiani *et al*. (2005), *E. coli* DNA polymerases Pol III and Pol IV can bind to one β clamp at the same time (Indiani *et al.*, 2005). Therefore, it has been hypothesized that MutL and Pol III may interact with β clamp in a similar way (Lopez de Saro *et al.*, 2006). Authors demonstrate that MutL competes with Pol III for binding to β clamp and suggest that when DNA polymerase has synthesized mismatch then MutL binds to ssDNA/dsDNA junction and displace Pol III core by direct competition for the binding to β clamp which may inhibit the ongoing DNA synthesis by polymerase as induces its backward movement to the place of mismatch for a proofreading activity. The DNA fragment could be then excised up to the mismatch. Also, drawing parallels from studies of eukaryotic MMR, MutL could recruit exonuclease and UvrD helicase to the β processivity clamp for displacement the newly synthesized strand (Dzantiev *et al.*, 2004). Thus, as *P. putida* MMR resembles to eukaryotic MMR in the lack of methylation of nascent DNA as well as the lack of MutH-provided function, then MutL endonucleolytic activity and its interaction with β clamp in *P. putida* may be sufficient for the strand nicking and subsequent excision. Considering this, when the above-mentioned interactions do not take place then downstream repair events are abolished and lesion in DNA persists regardless of its recognition by MutS. Hence, as accumulating evidences suggest, the processes occurring in *P. putida* are likely to be different from those characterized in the model organism *E. coli* and therefore the action of various components of MMR during DNA repair may not be entirely compatible to those reported in *E. coli*.

CONCLUSIONS

Bacteria spend majority of their lifetime under stressful conditions due to competition for available nutrients. Genetic adaptation of bacterial population often occurs under starvation conditions by mutations called either stationary phase mutations or stress-induced mutations. The process of stationary phase mutation encompasses mechanisms activated in response to stressful situations that collectively increase mutation rates and therefore genetic variability. So far, most of the research of stationary phase mutation has utilized *E. coli* model systems. Since the molecular mechanisms of stationary phase mutagenesis in non-enteric bacteria have remained poorly investigated, we have studied the molecular mechanisms underlying stress-induced mutational processes in the soil bacterium *Pseudomonas putida* as a model organism under stressful, carbon starvation conditions.

The main conclusions of the current dissertation are as follows:

1. Different mechanisms are responsible for the appearance of mutations in growing and starving cells of *P. putida.* These mechanisms change also during the time of starvation as the spectrum of stationary phase mutation among early arising mutants differed from that of late arising ones in case of particular test system used. While the C-to-A transversion was prominent mutation type during the first days of starvation, its proportion declined with the time of starvation and the percentage of 2- to 3-bp deletions and insertions increased. The generation of small deletions and insertions in starving *P. putida* required positive regulation conferred by stationary phase sigma factor RpoS.
2. Oxidative DNA damage is important source of stationary phase mutations in starving *P. putida*. The mutagenic effects of oxidized guanine are efficiently avoided by the DNA glycosylases of GO repair system that are functional during the starvation period studied. The inactivation of MutY or MutM enzyme enhanced the occurrence of G-to-T transversions in starving bacteria thereby confirming that the role of these DNA glycosylases in *P. putida* resembles to that of the respective glycosylases in *E. coli*. The absence of DNA glycosylase MutY enhanced base substitution mutation frequency independently of the growth phase of *P. putida*. In contrast, the deficiency of MutM glycosylase did not remarkably enhance mutation frequency neither in growing nor starving cells compared to wild type strain. However, the proportion of G-to-T transversions was significantly increased among stationary phase mutations in the absence of MutM, thus referring to the functionality of MutM in starving bacteria. We speculate that the activity of some other DNA glycosylase, possibly Nth homologue, could suppress the expected high mutation frequency in case MutM fails to be operative.

3. The antimutator effect of the nucleotide pool sanitation enzyme pyrophosphohydrolase MutT depended on the growth phase of *P. putida*. Although the lack of the MutT activity revealed strong mutator phenotype in starving bacteria with enhanced frequency of A-to-C transversions, it had only minor effect on the mutation frequency in growing bacteria. We hypothesize that mutator phenotype of MutT-defective bacteria in growing cells must be suppressed by backup enzymes, possibly by other Nudix hydrolases.
4. In addition to oxidized guanine, the oxidation products of adenine in DNA may contribute to the list of mutagens important in stationary phase mutation in starving bacteria. However, we did not detect the contribution of guanine secondary oxidation products to mutagenesis.
5. Stationary-phase-specific DNA binding protein Dps, which is known to protect genome from oxidative damage, has no role in the avoidance of stationary phase mutations in *P. putida* during starvation. Yet, Dps protects the genome of *P. putida* against exogenous oxidative DNA-damaging agents.
6. One of the widely discussed idea of the decline in DNA mismatch repair as the mechanism of the occurrence of stationary phase mutations in carbon-starved bacteria is not entirely compatible in *P. putida*. Lack of the MMR system in *P. putida* increased the emergence of stationary phase base substitution mutations about 25-fold in starving bacteria. The frequency of accumulation of base substitution mutants did not decline in starved populations of MMR-defective strains throughout the 2-week starvation period studied. This result strongly suggests that MMR is functional in repairing base substitutions during the time of starvation. Analysis of mutation spectra supported our suggestion of functionality of MMR throughout the starvation period as the spectra of MMR-defective strains were distinct from that of wild type strain. Thus, other mechanisms than malfunctioning of MMR system in starved cells must be considered to explain the accumulation of stationary phase mutations in *P. putida*.

Consequently, the data presented in the current dissertation suggest that the population of starving *P. putida* is dynamic and mechanisms of stationary phase mutation change also during starvation. Mutational processes underlying stationary phase mutation seem not to include the malfunctioning of DNA repair as a major mechanism of stationary phase mutation. However, as all processes leading to stationary phase mutation are someway connected to each other, the results presented in this dissertation encompass only one part of the complex machinery operating on mutagenesis. Thus, unravelling the complex network underlying the multifaceted nature of mechanisms of stationary phase mutation is a challenge for future studies.

LITERATURE CITED

Abella, M., Erill, I., Jara, M., Mazon, G., Campoy, S., and Barbe, J. (2004) Widespread distribution of a lexA-regulated DNA damage-inducible multiple gene cassette in the *Proteobacteria* phylum. *Mol Microbiol* **54**: 212–222.

Abeygunawardana, C., Weber, D.J., Gittis, A.G., Frick, D.N., Lin, J., Miller, A.F., Bessman, M.J., and Mildvan, A.S. (1995) Solution structure of the MutT enzyme, a nucleoside triphosphate pyrophosphohydrolase. *Biochemistry* **34**: 14997–15005.

Acharya, S., Foster, P.L., Brooks, P., and Fishel, R. (2003) The coordinated functions of the *E. coli* MutS and MutL proteins in mismatch repair. *Mol Cell* **12**: 233–246.

Aertsen, A., and Michiels, C.W. (2006) Upstream of the SOS response: figure out the trigger. *Trends Microbiol* **14**: 421–423.

Akasaka, S., and Yamamoto, K. (1994a) Hydrogen peroxide induces G:C to T:A and G:C to C:G transversions in the *supF* gene of *Escherichia coli*. *Mol Gen Genet* **243**: 500–505.

Akasaka, S., and Yamamoto, K. (1994b) Mutagenesis resulting from DNA damage by lipid peroxidation in the *supF* gene of *Escherichia coli*. *Mutat Res* **315**: 105–112.

Akasaka, S., and Yamamoto, K. (1995) Mutational specificity of the ferrous ion in a *supF* gene of *Escherichia coli*. *Biochem Biophys Res Commun* **213**: 74–80.

Ali Azam, T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. (1999) Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. *J Bacteriol* **181**: 6361–6370.

Ali, J.A., and Lohman, T.M. (1997) Kinetic measurement of the step size of DNA unwinding by *Escherichia coli* UvrD helicase. *Science.* **275**: 377–380.

Allen, D.J., Makhov, A., Grilley, M., Taylor, J., Thresher, R., Modrich, P., and Griffith, J.D. (1997) MutS mediates heteroduplex loop formation by a translocation mechanism. *EMBO J* **16**: 4467–4476.

Almiron, M., Link, A.J., Furlong, D., and Kolter, R. (1992) A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. *Genes Dev.* **6**: 2646–2654.

Altuvia, S., Almiron, M., Huisman, G., Kolter, R., and Storz, G. (1994) The *dps* promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. *Mol Microbiol* **13**: 265–272.

Andersson, D.I., Slechta, E.S., and Roth, J.R. (1998) Evidence that gene amplification underlies adaptive mutability of the bacterial *lac* operon. *Science* **282**: 1133–1135.

Andrews, S.C., Robinson, A.K., and Rodriguez-Quinones, F. (2003) Bacterial iron homeostasis. *FEMS Microbiol Rev* **27**: 215–237.

Aravind, L., and Koonin, E.V. (2001) Prokaryotic homologs of the eukaryotic DNA-end-binding protein Ku, novel domains in the Ku protein and prediction of a prokaryotic double-strand break repair system. *Genome Res* **11**: 1365–1374.

Asahara, H., Wistort, P.M., Bank, J.F., Bakerian, R.H., and Cunningham, R.P. (1989) Purification and characterization of *Escherichia coli* endonuclease III from the cloned *nth* gene. *Biochemistry* **28**: 4444–4449.

Azam, T.A., Hiraga, S., and Ishihama, A. (2000) Two types of localization of the DNA-binding proteins within the *Escherichia coli* nucleoid. *Genes Cells* **5**: 613–626.

23

Au, K.G., Cabrera, M., Miller, J.H., and Modrich, P. (1988) *Escherichia coli mutY* gene product is required for specific A-G----C.G mismatch correction. *Proc Natl Acad Sci U S A* **85**: 9163–9166.

Au, K.G., Welsh, K., and Modrich, P. (1992) Initiation of methyl-directed mismatch repair. *J Biol Chem* **267**: 12142–12148.

Baaghil, S., Lewin, A., Moore, G.R., and Le Brun, N.E. (2003) Core formation in *Escherichia coli* bacterioferritin requires a functional ferroxidase center. *Biochemistry* **42**: 14047–14056.

Bai, H., and Lu, A.L. (2007) Physical and functional interactions between *Escherichia coli* MutY glycosylase and mismatch repair protein MutS. *J Bacteriol* **189**: 902–910.

Bailly, V., and Verly, W.G. (1989) The multiple activities of *Escherichia coli* endonuclease IV and the extreme lability of 5'-terminal base-free deoxyribose 5-phosphates. *Biochem J* **259**: 761–768.

Bailly, V., Verly, W.G., O'Connor, T., and Laval, J. (1989) Mechanism of DNA strand nicking at apurinic/apyrimidinic sites by *Escherichia coli* [formamidopyrimidine]DNA glycosylase. *Biochem J* **262**: 581–589.

Balbinder, E., Coll, B., Hutchinson, J., Bianchi, A.S., Groman, T., Wheeler, K.A., and Meyer, M. (1993) Participation of the SOS system in producing deletions in *E. coli* plasmids. *Mutat Res.* **286**: 253–265.

Banerjee, A., Santos, W.L., and Verdine, G.L. (2006) Structure of a DNA glycosylase searching for lesions. *Science.* **311**: 1153–1157.

Barnes, D.E., Lindahl, T., and Sedgwick, B. (1993) DNA repair. *Curr Opin Cell Biol* **5**: 424–433.

Barone, F., McCulloch, S.D., Macpherson, P., Maga, G., Yamada, M., Nohmi, T., Minoprio, A., Mazzei, F., Kunkel, T.A., Karran, P., and Bignami, M. (2007) Replication of 2-hydroxyadenine-containing DNA and recognition by human MutSalpha. *DNA Repair* **6**: 355–366.

Barrett, T.E., Savva, R., Panayotou, G., Barlow, T., Brown, T., Jiricny, J., and Pearl, L.H. (1998) Crystal structure of a G:T/U mismatch-specific DNA glycosylase: mismatch recognition by complementary-strand interactions. *Cell* **92**: 117–129.

Bernards, A.S., Miller, J.K., Bao, K.K., and Wong, I. (2002) Flipping duplex DNA inside out: a double base-flipping reaction mechanism by *Escherichia coli* MutY adenine glycosylase. *J Biol Chem* **277**: 20960–20964.

Bessho, T., Tano, K., Kasai, H., and Nishimura, S. (1992) Deficiency of 8-hydroxyguanine DNA endonuclease activity and accumulation of the 8-hydroxyguanine in mutator mutant (*mutM*) of *Escherichia coli*. *Biochem Biophys Res Commun* **188**: 372–378.

Bessman, M.J., Frick, D.N., and O'Handley, S.F. (1996) The MutT proteins or "Nudix" hydrolases, a family of versatile, widely distributed, "housecleaning" enzymes. *J Biol Chem* **271**: 25059–25062.

Bhamre, S., Gadea, B.B., Koyama, C.A., White, S.J., and Fowler, R.G. (2001) An aerobic *recA*-, *umuC*-dependent pathway of spontaneous base-pair substitution mutagenesis in *Escherichia coli*. *Mutat Res.* **473**: 229–247.

Bhatnagar, S.K., and Bessman, M.J. (1988) Studies on the mutator gene, *mutT* of *Escherichia coli*. Molecular cloning of the gene, purification of the gene product, and identification of a novel nucleoside triphosphatase. *J Biol Chem* **263**: 8953–8957.

Bhatnagar, S.K., Bullions, L.C., and Bessman, M.J. (1991) Characterization of the *mutT* nucleoside triphosphatase of *Escherichia coli*. *J Biol Chem* **266**: 9050–9054.

Bjedov, I., Tenaillon, O., Gerard, B., Souza, V., Denamur, E., Radman, M., Taddei, F., and Matic, I. (2003) Stress-induced mutagenesis in bacteria. *Science* **300**: 1404–1409.

Bjelland, S., Birkeland, N.K., Benneche, T., Volden, G., and Seeberg, E. (1994) DNA glycosylase activities for thymine residues oxidized in the methyl group are functions of the AlkA enzyme in *Escherichia coli*. *J Biol Chem* **269**: 30489–30495.

Bjelland, S., and Seeberg, E. (1996) Different efficiencies of the Tag and AlkA DNA glycosylases from *Escherichia coli* in the removal of 3-methyladenine from single-stranded DNA. *FEBS Lett* **397**: 127–129.

Bjelland, S., and Seeberg, E. (2003) Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. *Mutat Res* **531**: 37–80.

Blackwell, L.J., Martik, D., Bjornson, K.P., Bjornson, E.S., and Modrich, P. (1998) Nucleotide-promoted release of hMutSalpha from heteroduplex DNA is consistent with an ATP-dependent translocation mechanism. *J Biol Chem* **273**: 32055–32062.

Blackwell, L.J., Bjornson, K.P., Allen, D.J., and Modrich, P. (2001) Distinct MutS DNA-binding modes that are differentially modulated by ATP binding and hydrolysis. *J Biol Chem* **276**: 34339–34347.

Blaisdell, J.O., Hatahet, Z., and Wallace, S.S. (1999) A novel role for *Escherichia coli* endonuclease VIII in prevention of spontaneous G-->T transversions. *J Bacteriol* **181**: 6396–6402.

Boiteux, S., O'Connor, T.R., and Laval, J. (1987) Formamidopyrimidine-DNA glycosylase of *Escherichia coli*: cloning and sequencing of the *fpg* structural gene and overproduction of the protein. *EMBO J* **6**: 3177–3183.

Boiteux, S., Gajewski, E., Laval, J., and Dizdaroglu, M. (1992) Substrate specificity of the *Escherichia coli* Fpg protein (formamidopyrimidine-DNA glycosylase): excision of purine lesions in DNA produced by ionizing radiation or photosensitization. *Biochemistry* **31**: 106–110.

Boon, E.M., Livingston, A.L., Chmiel, N.H., David, S.S., and Barton, J.K. (2003) DNA-mediated charge transport for DNA repair. *Proc Natl Acad Sci USA* **100**: 12543–12547.

Bregeon, D., Doddridge, Z.A., You, H.J., Weiss, B., and Doetsch, P.W. (2003) Transcriptional mutagenesis induced by uracil and 8-oxoguanine in *Escherichia coli*. *Mol Cell* **12**: 959–970.

Breimer, L.H. (1984) Enzymatic excision from gamma-irradiated polydeoxyribonucleotides of adenine residues whose imidazole rings have been ruptured. *Nucleic Acids Res* **12**: 6359–6367.

Bridges, B.A. (1996) Elevated mutation rate in *mutT* bacteria during starvation: evidence for DNA turnover? *J Bacteriol.* **178**: 2709–2711.

Bridges, B.A., Sekiguchi, M., and Tajiri, T. (1996) Effect of *mutY* and *mutM/fpg*-1 mutations on starvation-associated mutation in *Escherichia coli*: implications for the role of 7,8-dihydro-8-oxoguanine. *Mol Gen Genet.* **251**: 352–357.

Bridges, B.A., and Timms, A.R. (1997) Mutation in *Escherichia coli* under starvation conditions: a new pathway leading to small deletions in strains defective in mismatch correction. *EMBO J.* **16**: 3349–3356.

Bridges, B.A. (1998) The role of DNA damage in stationary phase ('adaptive') mutation. *Mutat Res* **408**: 1–9.

Bridges, B.A., and Ereira, S. (1998) DNA synthesis and viability of a *mutT* derivative of *Escherichia coli* WP2 under conditions of amino acid starvation and relation to stationary-phase (adaptive) mutation. *J Bacteriol* **180**: 2906–2910.

Bridges, B.A., and Timms, A. (1998) Effect of endogenous carotenoids and defective RpoS sigma factor on spontaneous mutation under starvation conditions in *Escherichia coli*: evidence for the possible involvement of singlet oxygen. *Mutat Res.* **403**: 21–28.

Bridges, B.A., Foster, P.L., and Timms, A.R. (2001) Effect of endogenous carotenoids on "adaptive" mutation in *Escherichia coli* FC40. *Mutat Res.* **473**: 109–119.

Brown, E.W., LeClerc, J.E., Li, B., Payne, W.L., and Cebula, T.A. (2001) Phylogenetic evidence for horizontal transfer of *mutS* alleles among naturally occurring *Escherichia coli* strains. *J Bacteriol.* **183**: 1631–1644.

Bruner, S.D., Norman, D.P., and Verdine, G.L. (2000) Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. *Nature* **403**: 859–866.

Bull, H.J., Lombardo, M.J., and Rosenberg, S.M. (2001) Stationary-phase mutation in the bacterial chromosome: recombination protein and DNA polymerase IV dependence. *Proc Natl Acad Sci USA.* **98**: 8334–8341.

Burgess, S., Jaruga, P., Dodson, M.L., Dizdaroglu, M., and Lloyd, R.S. (2002) Determination of active site residues in Escherichia coli endonuclease VIII. *J Biol Chem* **277**: 2938–2944.

Burrows, C.J., and Muller, J.G. (1998) Oxidative Nucleobase Modifications Leading to Strand Scission. *Chem Rev* **98**: 1109–1152.

Cabrera, M., Nghiem, Y., and Miller, J.H. (1988) mutM, a second mutator locus in Escherichia coli that generates G.C----T.A transversions. *J Bacteriol* **170**: 5405–5407.

Cadet, J., Delatour, T., Douki, T., Gasparutto, D., Pouget, J.P., Ravanat, J.L., and Sauvaigo, S. (1999) Hydroxyl radicals and DNA base damage. *Mutat Res* **424**: 9–21.

Cairns, J., Overbaugh, J., and Miller, S. (1988) The origin of mutants. *Nature* **335**: 142–145.

Cairns, J., and Foster, P.L. (1991) Adaptive reversion of a frameshift mutation in *Escherichia coli*. *Genetics* **128**: 695–701.

Ceci, P., Ilari, A., Falvo, E., and Chiancone, E. (2003) The Dps protein of *Agrobacterium tumefaciens* does not bind to DNA but protects it toward oxidative cleavage: x-ray crystal structure, iron binding, and hydroxyl-radical scavenging properties. *J Biol Chem* **278**: 20319–20326.

Ceci, P., Cellai, S., Falvo, E., Rivetti, C., Rossi, G.L., and Chiancone, E. (2004) DNA condensation and self-aggregation of *Escherichia coli* Dps are coupled phenomena related to the properties of the N-terminus. *Nucleic Acids Res* **32**: 5935–5944.

Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., and Loeb, L.A. (1992) 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions. *J Biol Chem* **267**: 166–172.

Chetsanga, C.J., and Lindahl, T. (1979) Release of 7-methylguanine residues whose imidazole rings have been opened from damaged DNA by a DNA glycosylase from *Escherichia coli*. *Nucleic Acids Res* **6**: 3673–3684.

Choi, S.H., Baumler, D.J., and Kaspar, C.W. (2000) Contribution of *dps* to acid stress tolerance and oxidative stress tolerance in *Escherichia coli* O157:H7. *Appl Environ Microbiol* **66**: 3911–3916.

Clark, A.J., and Margulies, A.D. (1965) Isolation and Characterization of Recombination-Deficient Mutants of *Escherichia coli* K12. *Proc Natl Acad Sci USA* **53**: 451–459.

Clarke, N.D., Kvaal, M., and Seeberg, E. (1984) Cloning of *Escherichia coli* genes encoding 3-methyladenine DNA glycosylases I and II. *Mol Gen Genet* **197**: 368–372.

Cooke, M.S., Evans, M.D., Dizdaroglu, M., and Lunec, J. (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *Faseb J.* **17**: 1195–1214.

Courcelle, J., Khodursky, A., Peter, B., Brown, P.O., and Hanawalt, P.C. (2001) Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli*. *Genetics* **158**: 41–64.

Cromie, G.A., Connelly, J.C., and Leach, D.R. (2001) Recombination at double-strand breaks and DNA ends: conserved mechanisms from phage to humans. *Mol Cell* **8**: 1163–1174.

Cunningham, R.P., and Weiss, B. (1985) Endonuclease III (*nth*) mutants of *Escherichia coli*. *Proc Natl Acad Sci U S A* **82**: 474–478.

Cunningham, R.P., Asahara, H., Bank, J.F., Scholes, C.P., Salerno, J.C., Surerus, K., Munck, E., McCracken, J., Peisach, J., and Emptage, M.H. (1989) Endonuclease III is an iron-sulfur protein. *Biochemistry* **28**: 4450–4455.

Cupples, C.G., and Miller, J.H. (1989) A set of *lacZ* mutations in *Escherichia coli* that allow rapid detection of each of the six base substitutions. *Proc Natl Acad Sci USA* **86**: 5345–5349.

D'Ham, C., Romieu, A., Jaquinod, M., Gasparutto, D., and Cadet, J. (1999) Excision of 5,6-dihydroxy-5,6-dihydrothymine, 5,6-dihydrothymine, and 5-hydroxycytosine from defined sequence oligonucleotides by *Escherichia coli* endonuclease III and Fpg proteins: kinetic and mechanistic aspects. *Biochemistry* **38**: 3335–3344.

Dalrymple, B.P., Kongsuwan, K., Wijffels, G., Dixon, N.E., and Jennings, P.A. (2001) A universal protein-protein interaction motif in the eubacterial DNA replication and repair systems. *Proc Natl Acad Sci USA*. **98**: 11627–11632.

Dao, V., and Modrich, P. (1998) Mismatch-, MutS-, MutL-, and helicase II-dependent unwinding from the single-strand break of an incised heteroduplex. *J Biol Chem* **273**: 9202–9207.

David-Cordonnier, M.H., Laval, J., and O'Neill, P. (2000) Clustered DNA damage, influence on damage excision by XRS5 nuclear extracts and Escherichia coli Nth and Fpg proteins. *J Biol Chem* **275**: 11865–11873.

David, S.S., and Williams, S.D. (1998) Chemistry of Glycosylases and Endonucleases Involved in Base-Excision Repair. *Chem Rev* **98**: 1221–1262.

David, S.S., O'Shea, V.L., and Kundu, S. (2007) Base-excision repair of oxidative DNA damage. *Nature* **447**: 941–950.

Davidsen, T., Bjoras, M., Seeberg, E.C., and Tonjum, T. (2005) Antimutator role of DNA glycosylase MutY in pathogenic *Neisseria* species. *J Bacteriol* **187**: 2801–2809.

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Davidsen, T., Tuven, H.K., Bjoras, M., Rodland, E.A., and Tonjum, T. (2007) Genetic Interactions of DNA Repair Pathways in the Pathogen *Neisseria meningitidis*. *J Bacteriol* **189**: 5728–5737.

de Weert, S., Dekkers, L.C., Kuiper, I., Bloemberg, G.V., and Lugtenberg, B.J. (2004) Generation of enhanced competitive root-tip-colonizing *Pseudomonas* bacteria through accelerated evolution. *J Bacteriol* **186**: 3153–3159.

Dedon, P.C., and Tannenbaum, S.R. (2004) Reactive nitrogen species in the chemical biology of inflammation. *Arch Biochem Biophys* **423**: 12–22.

Delaney, S., Neeley, W.L., Delaney, J.C., and Essigmann, J.M. (2007) The substrate specificity of MutY for hyperoxidized guanine lesions in vivo. *Biochemistry* **46**: 1448–1455.

Demple, B., and Linn, S. (1980) DNA N-glycosylases and UV repair. *Nature* **287**: 203–208.

Demple, B., and Harrison, L. (1994) Repair of oxidative damage to DNA: enzymology and biology. *Annu Rev Biochem* **63**: 915–948.

Dianov, G., Sedgwick, B., Daly, G., Olsson, M., Lovett, S., and Lindahl, T. (1994) Release of 5'-terminal deoxyribose-phosphate residues from incised abasic sites in DNA by the *Escherichia coli* RecJ protein. *Nucleic Acids Res* **22**: 993–998.

Dizdaroglu, M. (1992) Oxidative damage to DNA in mammalian chromatin. *Mutat Res* **275**: 331–342.

Dizdaroglu, M., Laval, J., and Boiteux, S. (1993) Substrate specificity of the *Escherichia coli* endonuclease III: excision of thymine- and cytosine-derived lesions in DNA produced by radiation-generated free radicals. *Biochemistry* **32**: 12105–12111.

Dizdaroglu, M., Bauche, C., Rodriguez, H., and Laval, J. (2000) Novel substrates of *Escherichia coli* Nth protein and its kinetics for excision of modified bases from DNA damaged by free radicals. *Biochemistry* **39**: 5586–5592.

Dizdaroglu, M., Burgess, S.M., Jaruga, P., Hazra, T.K., Rodriguez, H., and Lloyd, R.S. (2001) Substrate specificity and excision kinetics of *Escherichia coli* endonuclease VIII (Nei) for modified bases in DNA damaged by free radicals. *Biochemistry* **40**: 12150–12156.

Dizdaroglu, M. (2005) Base-excision repair of oxidative DNA damage by DNA glycosylases. *Mutat Res* **591**: 45–59.

Drake, J.W. (1999) The distribution of rates of spontaneous mutation over viruses, prokaryotes, and eukaryotes. *Ann N Y Acad Sci* **870**: 100–107.

Dzantiev, L., Constantin, N., Genschel, J., Iyer, R.R., Burgers, P.M., and Modrich, P. (2004) A defined human system that supports bidirectional mismatch-provoked excision. *Mol Cell* **15**: 31–41.

Duarte, V., Gasparutto, D., Jaquinod, M., and Cadet, J. (2000) In vitro DNA synthesis opposite oxazolone and repair of this DNA damage using modified oligonucleotides. *Nucleic Acids Res* **28**: 1555–1563.

Duarte, V., Gasparutto, D., Jaquinod, M., Ravanat, J., and Cadet, J. (2001) Repair and mutagenic potential of oxaluric acid, a major product of singlet oxygen-mediated oxidation of 8-oxo-7,8-dihydroguanine. *Chem Res Toxicol* **14**: 46–53.

Einolf, H.J., Schnetz-Boutaud, N., and Guengerich, F.P. (1998) Steady-state and pre-steady-state kinetic analysis of 8-oxo-7,8-dihydroguanosine triphosphate incorporation and extension by replicative and repair DNA polymerases. *Biochemistry.* **37**: 13300–13312.

Einolf, H.J., and Guengerich, F.P. (2001) Fidelity of nucleotide insertion at 8-oxo-7,8-dihydroguanine by mammalian DNA polymerase delta. Steady-state and pre-steady-state kinetic analysis. *J Biol Chem* **276**: 3764–3771.

Erill, I., Campoy, S., Mazon, G., and Barbe, J. (2006) Dispersal and regulation of an adaptive mutagenesis cassette in the bacteria domain. *Nucleic Acids Res* **34**: 66–77.

Falnes, P.O., Johansen, R.F., and Seeberg, E. (2002) AlkB-mediated oxidative demethylation reverses DNA damage in *Escherichia coli*. *Nature* **419**: 178–182.

Feng, G., Tsui, H.C., and Winkler, M.E. (1996) Depletion of the cellular amounts of the MutS and MutH methyl-directed mismatch repair proteins in stationary-phase *Escherichia coli* K-12 cells. *J Bacteriol* **178**: 2388–2396.

Feng, W.Y., Lee, E.H., and Hays, J.B. (1991) Recombinagenic processing of UV-light photoproducts in nonreplicating phage DNA by the *Escherichia coli* methyl-directed mismatch repair system. *Genetics* **129**: 1007–1020.

Finkel, S.E. (2006) Long-term survival during stationary phase: evolution and the GASP phenotype. *Nat Rev Microbiol.* **4**: 113–120.

Flint, D.H., Emptage, M.H., Finnegan, M.G., Fu, W., and Johnson, M.K. (1993) The role and properties of the iron-sulfur cluster in *Escherichia coli* dihydroxy-acid dehydratase. *J Biol Chem* **268**: 14732–14742.

Foster, P.L. (1992) Directed mutation: between unicorns and goats. *J Bacteriol* **174**: 1711–1716.

Foster, P.L., and Trimarchi, J.M. (1994) Adaptive reversion of a frameshift mutation in *Escherichia coli* by simple base deletions in homopolymeric runs. *Science* **265**: 407–409.

Foster, P.L., Gudmundsson, G., Trimarchi, J.M., Cai, H., and Goodman, M.F. (1995) Proofreading-defective DNA polymerase II increases adaptive mutation in *Escherichia coli*. *Proc Natl Acad Sci USA* **92**: 7951–7955.

Foster, P.L., and Trimarchi, J.M. (1995a) Adaptive reversion of an episomal frameshift mutation in *Escherichia coli* requires conjugal functions but not actual conjugation. *Proc Natl Acad Sci USA*. **92**: 5487–5490.

Foster, P.L., and Trimarchi, J.M. (1995b) Conjugation is not required for adaptive reversion of an episomal frameshift mutation in *Escherichia coli*. *J Bacteriol.* **177**: 6670–6671.

Foster, P.L., Trimarchi, J.M., and Maurer, R.A. (1996) Two enzymes, both of which process recombination intermediates, have opposite effects on adaptive mutation in *Escherichia coli*. *Genetics* **142**: 25–37.

Foster, P.L. (1997) Nonadaptive mutations occur on the F' episome during adaptive mutation conditions in *Escherichia coli*. *J Bacteriol* **179**: 1550–1554.

Foster, P.L. (1999a) Mechanisms of stationary phase mutation: a decade of adaptive mutation. *Annu Rev Genet* **33**: 57–88.

Foster, P.L. (1999b) Are adaptive mutations due to a decline in mismatch repair? The evidence is lacking. *Mutat Res* **436**: 179–184.

Foster, P.L. (2000) Adaptive mutation: implications for evolution. *Bioessays* **22**: 1067–1074.

Foster, P.L. (2004) Adaptive mutation in *Escherichia coli*. *J Bacteriol.* **186**: 4846–4852.

Foster, P.L. (2005) Stress responses and genetic variation in bacteria. *Mutat Res* **569**: 3–11.

Fowler, R.G., and Schaaper, R.M. (1997) The role of the *mutT* gene of *Escherichia coli* in maintaining replication fidelity. *FEMS Microbiol Rev* **21**: 43–54.

Frelon, S., Douki, T., and Cadet, J. (2002) Radical oxidation of the adenine moiety of nucleoside and DNA: 2-hydroxy-2'-deoxyadenosine is a minor decomposition product. *Free Radic Res* **36**: 499–508.

Frenkiel-Krispin, D., Levin-Zaidman, S., Shimoni, E., Wolf, S.G., Wachtel, E.J., Arad, T., Finkel, S.E., Kolter, R., and Minsky, A. (2001) Regulated phase transitions of bacterial chromatin: a non-enzymatic pathway for generic DNA protection. *EMBO J* **20**: 1184–1191.

Frenkiel-Krispin, D., Ben-Avraham, I., Englander, J., Shimoni, E., Wolf, S.G., and Minsky, A. (2004) Nucleoid restructuring in stationary-state bacteria. *Mol Microbiol* **51**: 395–405.

Frenkiel-Krispin, D., and Minsky, A. (2006) Nucleoid organization and the maintenance of DNA integrity in *E. coli*, *B. subtilis* and *D. radiodurans*. *J Struct Biol* **156**: 311–319.

Fridovich, I. (1995) Superoxide radical and superoxide dismutases. *Annu Rev Biochem* **64**: 97–112.

Friedberg, E.C., Feaver, W.J., and Gerlach, V.L. (2000) The many faces of DNA polymerases: strategies for mutagenesis and for mutational avoidance. *Proc Natl Acad Sci USA* **97**: 5681–5683.

Friedberg, E.C., Wagner, R., and Radman, M. (2002) Specialized DNA polymerases, cellular survival, and the genesis of mutations. *Science* **296**: 1627–1630.

Friedberg, E.C. (2003) DNA damage and repair. *Nature.* **421**: 436–440.

Friedberg, E.C., McDaniel, L.D., and Schultz, R.A. (2004) The role of endogenous and exogenous DNA damage and mutagenesis. *Curr Opin Genet Dev* **14**: 5–10.

Friedberg, E.C. (2005) Suffering in silence: the tolerance of DNA damage. *Nat Rev Mol Cell Biol* **6**: 943–953.

Friedberg, E.C., Lehmann, A.R., and Fuchs, R.P. (2005) Trading places: how do DNA polymerases switch during translesion DNA synthesis? *Mol Cell.* **18**: 499–505.

Fromme, J.C., and Verdine, G.L. (2002) Structural insights into lesion recognition and repair by the bacterial 8-oxoguanine DNA glycosylase MutM. *Nat Struct Biol.* **9**: 544–552.

Fromme, J.C., and Verdine, G.L. (2003) Structure of a trapped endonuclease III-DNA covalent intermediate. *EMBO J.* **22**: 3461–3471.

Fromme, J.C., Banerjee, A., Huang, S.J., and Verdine, G.L. (2004a) Structural basis for removal of adenine mispaired with 8-oxoguanine by MutY adenine DNA glycosylase. *Nature.* **427**: 652–656.

Fromme, J.C., Banerjee, A., and Verdine, G.L. (2004b) DNA glycosylase recognition and catalysis. *Curr Opin Struct Biol.* **14**: 43–49.

Fujikawa, K., and Kasai, H. (2002) The oxidized pyrimidine ribonucleotide, 5-hydroxy-CTP, is hydrolyzed efficiently by the *Escherichia coli* recombinant Orf135 protein. *DNA Repair (Amst)* **1**: 571–576.

Gabelli, S.B., Bianchet, M.A., Bessman, M.J., and Amzel, L.M. (2001) The structure of ADP-ribose pyrophosphatase reveals the structural basis for the versatility of the Nudix family. *Nat Struct Biol* **8**: 467–472.

Galhardo, R.S., Rocha, R.P., Marques, M.V., and Menck, C.F. (2005) An SOS-regulated operon involved in damage-inducible mutagenesis in *Caulobacter crescentus*. *Nucleic Acids Res* **33**: 2603–2614.

Galitski, T., and Roth, J.R. (1995) Evidence that F plasmid transfer replication underlies apparent adaptive mutation. *Science* **268**: 421–423.

Gallinari, P., and Jiricny, J. (1996) A new class of uracil-DNA glycosylases related to human thymine-DNA glycosylase. *Nature* **383**: 735–738.

Galperin, M.Y., Moroz, O.V., Wilson, K.S., and Murzin, A.G. (2006) House cleaning, a part of good housekeeping. *Mol Microbiol* **59**: 5–19.

Garibyan, L., Huang, T., Kim, M., Wolff, E., Nguyen, A., Nguyen, T., Diep, A., Hu, K., Iverson, A., Yang, H., and Miller, J.H. (2003) Use of the *rpoB* gene to determine the specificity of base substitution mutations on the *Escherichia coli* chromosome. *DNA Repair (Amst)* **2**: 593–608.

Gasparutto, D., Da Cruz, S., Bourdat, A.G., Jaquinod, M., and Cadet, J. (1999) Synthesis and biochemical properties of cyanuric acid nucleoside-containing DNA oligomers. *Chem Res Toxicol* **12**: 630–638.

Gentil, A., Cabral-Neto, J.B., Mariage-Samson, R., Margot, A., Imbach, J.L., Rayner, B., and Sarasin, A. (1992) Mutagenicity of a unique apurinic/apyrimidinic site in mammalian cells. *J Mol Biol* **227**: 981–984.

Gilboa, R., Zharkov, D.O., Golan, G., Fernandes, A.S., Gerchman, S.E., Matz, E., Kycia, J.H., Grollman, A.P., and Shoham, G. (2002) Structure of formamidopyrimidine-DNA glycosylase covalently complexed to DNA. *J Biol Chem* **277**: 19811–19816.

Godoy, V.G., Gizatullin, F.S., and Fox, M.S. (2000) Some features of the mutability of bacteria during nonlethal selection. *Genetics.* **154**: 49–59.

Gogos, A., Cillo, J., Clarke, N.D., and Lu, A.L. (1996) Specific recognition of A/G and A/7,8-dihydro-8-oxoguanine (8-oxoG) mismatches by *Escherichia coli* MutY: removal of the C-terminal domain preferentially affects A/8-oxoG recognition. *Biochemistry* **35**: 16665–16671.

Goodman, M.F. (2002) Error-prone repair DNA polymerases in prokaryotes and eukaryotes. *Annu Rev Biochem* **71**: 17–50.

Gradia, S., Acharya, S., and Fishel, R. (1997) The human mismatch recognition complex hMSH2-hMSH6 functions as a novel molecular switch. *Cell* **91**: 995–1005.

Gradia, S., Subramanian, D., Wilson, T., Acharya, S., Makhov, A., Griffith, J., and Fishel, R. (1999) hMSH2-hMSH6 forms a hydrolysis-independent sliding clamp on mismatched DNA. *Mol Cell* **3**: 255–261.

Gradia, S., Acharya, S., and Fishel, R. (2000) The role of mismatched nucleotides in activating the hMSH2-hMSH6 molecular switch. *J Biol Chem* **275**: 3922–3930.

Grant, R.A., Filman, D.J., Finkel, S.E., Kolter, R., and Hogle, J.M. (1998) The crystal structure of Dps, a ferritin homolog that binds and protects DNA. *Nat Struct Biol* **5**: 294–303.

Graves, R.J., Felzenszwalb, I., Laval, J., and O'Connor, T.R. (1992) Excision of 5'-terminal deoxyribose phosphate from damaged DNA is catalyzed by the Fpg protein of *Escherichia coli*. *J Biol Chem* **267**: 14429–14435.

Greated, A., Lambertsen, L., Williams, P.A., and Thomas, C.M. (2002) Complete sequence of the IncP-9 TOL plasmid pWW0 from *Pseudomonas putida*. *Environ Microbiol* **4**: 856–871.

Grilley, M., Griffith, J., and Modrich, P. (1993) Bidirectional excision in methyl-directed mismatch repair. *J Biol Chem* **268**: 11830–11837.
Grollman, A.P., and Moriya, M. (1993) Mutagenesis by 8-oxoguanine: an enemy within. *Trends Genet* **9**: 246–249.
Gros, L., Saparbaev, M.K., and Laval, J. (2002) Enzymology of the repair of free radicals-induced DNA damage. *Oncogene* **21**: 8905–8925.
Grzesiuk, E., Gozdek, A., and Tudek, B. (2001) Contribution of *E. coli* AlkA, TagA glycosylases and UvrABC-excinuclease in MMS mutagenesis. *Mutat Res* **480–481**: 77–84.
Guan, Y., Manuel, R.C., Arvai, A.S., Parikh, S.S., Mol, C.D., Miller, J.H., Lloyd, S., and Tainer, J.A. (1998) MutY catalytic core, mutant and bound adenine structures define specificity for DNA repair enzyme superfamily. *Nat Struct Biol.* **5**: 1058–1064.
Hailer, M.K., Slade, P.G., Martin, B.D., and Sugden, K.D. (2005) Nei deficient *Escherichia coli* are sensitive to chromate and accumulate the oxidized guanine lesion spiroiminodihydantoin. *Chem Res Toxicol* **18**: 1378–1383.
Hall, B.G. (1990) Spontaneous point mutations that occur more often when advantageous than when neutral. *Genetics* **126**: 5–16.
Hall, B.G. (1995) Genetics of selection-induced mutations: I. *uvrA*, *uvrB*, *uvrC*, and *uvrD* are selection-induced specific mutator loci. *J Mol Evol* **40**: 86–93.
Hall, M.C., Jordan, J.R., and Matson, S.W. (1998) Evidence for a physical interaction between the *Escherichia coli* methyl-directed mismatch repair proteins MutL and UvrD. *EMBO J* **17**: 1535–1541.
Hall, M.C., and Matson, S.W. (1999) The *Escherichia coli* MutL protein physically interacts with MutH and stimulates the MutH-associated endonuclease activity. *J Biol Chem* **274**: 1306–1312.
Hara, R., Mo, J., and Sancar, A. (2000) DNA damage in the nucleosome core is refractory to repair by human excision nuclease. *Mol Cell Biol* **20**: 9173–9181.
Harfe, B.D., and Jinks-Robertson, S. (2000) DNA mismatch repair and genetic instability. *Annu Rev Genet.* **34**: 359–399.
Harris, R.S., Longerich, S., and Rosenberg, S.M. (1994) Recombination in adaptive mutation. *Science* **264**: 258–260.
Harris, R.S., Ross, K.J., and Rosenberg, S.M. (1996) Opposing roles of the holliday junction processing systems of *Escherichia coli* in recombination-dependent adaptive mutation. *Genetics* **142**: 681–691.
Harris, R.S., Feng, G., Ross, K.J., Sidhu, R., Thulin, C., Longerich, S., Szigety, S.K., Winkler, M.E., and Rosenberg, S.M. (1997) Mismatch repair protein MutL becomes limiting during stationary-phase mutation. *Genes Dev* **11**: 2426–2437.
Harris, R.S., Feng, G., Ross, K.J., Sidhu, R., Thulin, C., Longerich, S., Szigety, S.K., Hastings, P.J., Winkler, M.E., and Rosenberg, S.M. (1999) Mismatch repair is diminished during stationary-phase mutation. *Mutat Res.* **437**: 51–60.
Hashiguchi, K., Zhang, Q.M., Sugiyama, H., Ikeda, S., and Yonei, S. (2002) Characterization of 2-hydroxyadenine DNA glycosylase activity of *Escherichia coli* MutY protein. *Int J Radiat Biol* **78**: 585–592.
Hastings, P.J., and Rosenberg, S.M. (2002) In pursuit of a molecular mechanism for adaptive gene amplification. *DNA Repair (Amst)* **1**: 111–123.

Hazra, T.K., Izumi, T., Venkataraman, R., Kow, Y.W., Dizdaroglu, M., and Mitra, S. (2000) Characterization of a novel 8-oxoguanine-DNA glycosylase activity in *Escherichia coli* and identification of the enzyme as endonuclease VIII. *J Biol Chem* **275**: 27762–27767.

Hazra, T.K., Hill, J.W., Izumi, T., and Mitra, S. (2001a) Multiple DNA glycosylases for repair of 8-oxoguanine and their potential in vivo functions. *Prog Nucleic Acid Res Mol Biol* **68**: 193–205.

Hazra, T.K., Muller, J.G., Manuel, R.C., Burrows, C.J., Lloyd, R.S., and Mitra, S. (2001b) Repair of hydantoins, one electron oxidation product of 8-oxoguanine, by DNA glycosylases of *Escherichia coli*. *Nucleic Acids Res* **29**: 1967–1974.

Hatahet, Z., Kow, Y.W., Purmal, A.A., Cunningham, R.P., and Wallace, S.S. (1994) New substrates for old enzymes. 5-Hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxyuridine are substrates for *Escherichia coli* endonuclease III and formamidopyrimidine DNA N-glycosylase, while 5-hydroxy-2'-deoxyuridine is a substrate for uracil DNA N-glycosylase. *J Biol Chem* **269**: 18814–18820.

He, A.S., Rohatgi, P.R., Hersh, M.N., and Rosenberg, S.M. (2006) Roles of *E. coli* double-strand-break-repair proteins in stress-induced mutation. *DNA Repair (Amst)* **5**: 258–273.

Heller, R.C., and Marians, K.J. (2006) Replication fork reactivation downstream of a blocked nascent leading strand. *Nature* **439**: 557–562.

Hempstead, P.D., Yewdall, S.J., Fernie, A.R., Lawson, D.M., Artymiuk, P.J., Rice, D.W., Ford, G.C., and Harrison, P.M. (1997) Comparison of the three-dimensional structures of recombinant human H and horse L ferritins at high resolution. *J Mol Biol* **268**: 424–448.

Henderson, P.T., Delaney, J.C., Gu, F., Tannenbaum, S.R., and Essigmann, J.M. (2002) Oxidation of 7,8-dihydro-8-oxoguanine affords lesions that are potent sources of replication errors in vivo. *Biochemistry.* **41**: 914–921.

Henderson, P.T., Delaney, J.C., Muller, J.G., Neeley, W.L., Tannenbaum, S.R., Burrows, C.J., and Essigmann, J.M. (2003) The hydantoin lesions formed from oxidation of 7,8-dihydro-8-oxoguanine are potent sources of replication errors in vivo. *Biochemistry* **42**: 9257–9262.

Henderson, P.T., Neeley, W.L., Delaney, J.C., Gu, F., Niles, J.C., Hah, S.S., Tannenbaum, S.R., and Essigmann, J.M. (2005) Urea lesion formation in DNA as a consequence of 7,8-dihydro-8-oxoguanine oxidation and hydrolysis provides a potent source of point mutations. *Chem Res Toxicol* **18**: 12–18.

Hendrickson, H., Slechta, E.S., Bergthorsson, U., Andersson, D.I., and Roth, J.R. (2002) Amplification-mutagenesis: evidence that "directed" adaptive mutation and general hypermutability result from growth with a selected gene amplification. *Proc Natl Acad Sci USA* **99**: 2164–2169. .

Hengge-Aronis, R. (2002) Signal Transduction and Regulatory Mechanisms Involved in Control of the sigma(S) (RpoS) Subunit of RNA Polymerase. *Microbiol Mol Biol Rev* **66**: 373–395.

Hersh, M.N., Ponder, R.G., Hastings, P.J., and Rosenberg, S.M. (2004) Adaptive mutation and amplification in *Escherichia coli*: two pathways of genome adaptation under stress. *Res Microbiol* **155**: 352–359.

Hogardt, M., Schubert, S., Adler, K., Gotzfried, M., and Heesemann, J. (2006) Sequence variability and functional analysis of MutS of hypermutable *Pseudomonas aeruginosa* cystic fibrosis isolates. *Int J Med Microbiol* **296**: 313–320.

Hopfner, K.P., and Tainer, J.A. (2000) DNA mismatch repair: the hands of a genome guardian. *Structure.* **8**: R237–241.

Hori, M., Fujikawa, K., Kasai, H., Harashima, H., and Kamiya, H. (2005) Dual hydrolysis of diphosphate and triphosphate derivatives of oxidized deoxyadenosine by Orf17 (NtpA), a MutT-type enzyme. *DNA Repair* **4**: 33–39.

Hori, M., Asanuma, T., Inanami, O., Kuwabara, M., Harashima, H., and Kamiya, H. (2006) Effects of Overexpression and Antisense RNA Expression of Orf17, a MutT-Type Enzyme. *Biol. Pharm. Bull.* **29**: 1087–1091.

Howard-Flanders, P., Boyce, R.P., and Theriot, L. (1966) Three loci in *Escherichia coli* K-12 that control the excision of pyrimidine dimers and certain other mutagen products from DNA. *Genetics* **53**: 1119–1136.

Hsieh, P. (2001) Molecular mechanisms of DNA mismatch repair. *Mutat Res.* **486**: 71–87.

Huffman, J.L., Sundheim, O., and Tainer, J.A. (2005) DNA base damage recognition and removal: new twists and grooves. *Mutat Res* **577**: 55–76.

Ilari, A., Stefanini, S., Chiancone, E., and Tsernoglou, D. (2000) The dodecameric ferritin from *Listeria innocua* contains a novel intersubunit iron-binding site. *Nat Struct Biol* **7**: 38–43.

Ilari, A., Ceci, P., Ferrari, D., Rossi, G.L., and Chiancone, E. (2002) Iron incorporation into *Escherichia coli* Dps gives rise to a ferritin-like microcrystalline core. *J Biol Chem* **277**: 37619–37623.

Ilves, H., Hõrak, R., and Kivisaar, M. (2001) Involvement of sigma(S) in starvation-induced transposition of *Pseudomonas putida* transposon Tn*4652*. *J Bacteriol* **183**: 5445–5448.

Imlay, J.A. (2002) What biological purpose is served by superoxide reductase? *J Biol Inorg Chem* **7**: 659–663.

Imlay, J.A. (2003) Pathways of oxidative damage. *Annu Rev Microbiol.* **57**: 395–418.

Indiani, C., McInerney, P., Georgescu, R., Goodman, M.F., and O'Donnell, M. (2005) A sliding-clamp toolbelt binds high- and low-fidelity DNA polymerases simultaneously. *Mol Cell* **19**: 805–815.

Inoue, M., Kamiya, H., Fujikawa, K., Ootsuyama, Y., Murata-Kamiya, N., Osaki, T., Yasumoto, K., and Kasai, H. (1998) Induction of chromosomal gene mutations in *Escherichia coli* by direct incorporation of oxidatively damaged nucleotides. New evaluation method for mutagenesis by damaged DNA precursors in vivo. *J Biol Chem.* **273**: 11069–11074.

Ishikawa, T., Mizunoe, Y., Kawabata, S., Takade, A., Harada, M., Wai, S.N., and Yoshida, S. (2003) The iron-binding protein Dps confers hydrogen peroxide stress resistance to *Campylobacter jejuni*. *J Bacteriol* **185**: 1010–1017.

Ito, R., Hayakawa, H., Sekiguchi, M., and Ishibashi, T. (2005) Multiple enzyme activities of *Escherichia coli* MutT protein for sanitization of DNA and RNA precursor pools. *Biochemistry* **44**: 6670–6674.

Iyer, R.R., Pluciennik, A., Burdett, V., and Modrich, P.L. (2006) DNA mismatch repair: functions and mechanisms. *Chem Rev* **106**: 302–323.

Jankovic, M., Kostic, T., and Savic, D.J. (1990) DNA sequence analysis of spontaneous histidine mutations in a *polA1* strain of *Escherichia coli* K12 suggests a specific role of the GTGG sequence. *Mol Gen Genet* **223**: 481–486.

Jeong, K.C., Baumler, D.J., and Kaspar, C.W. (2006) *dps* expression in *Escherichia coli* O157:H7 requires an extended –10 region and is affected by the cAMP receptor protein. *Biochim Biophys Acta* **1759**: 51–59.

Jiang, D., Hatahet, Z., Blaisdell, J.O., Melamede, R.J., and Wallace, S.S. (1997a) *Escherichia coli* endonuclease VIII: cloning, sequencing, and overexpression of the *nei* structural gene and characterization of *nei* and nei *nth* mutants. *J Bacteriol* **179**: 3773–3782.

Jiang, D., Hatahet, Z., Melamede, R.J., Kow, Y.W., and Wallace, S.S. (1997b) Characterization of *Escherichia coli* endonuclease VIII. *J Biol Chem* **272**: 32230–32239.

Jin, D.J., and Gross, C.A. (1988) Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. *J Mol Biol* **202**: 45–58.

Jiricny, J., Su, S.S., Wood, S.G., and Modrich, P. (1988) Mismatch-containing oligonucleotide duplexes bound by the *E. coli mutS*-encoded protein. *Nucleic Acids Res* **16**: 7843–7853.

Jiricny, J. (1998) Replication errors: cha(lle)nging the genome. *EMBO J* **17**: 6427–6436.

Jiricny, J. (2000) Mismatch repair: the praying hands of fidelity. *Curr Biol.* **10**: R788–790.

Jiricny, J. (2006) The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol* **7**: 335–346.

Junop, M.S., Obmolova, G., Rausch, K., Hsieh, P., and Yang, W. (2001) Composite active site of an ABC ATPase: MutS uses ATP to verify mismatch recognition and authorize DNA repair. *Mol Cell* **7**: 1–12.

Junop, M.S., Yang, W., Funchain, P., Clendenin, W., and Miller, J.H. (2003) In vitro and in vivo studies of MutS, MutL and MutH mutants: correlation of mismatch repair and DNA recombination. *DNA Repair (Amst)* **2**: 387–405.

Kadyrov, F.A., Dzantiev, L., Constantin, N., and Modrich, P. (2006) Endonucleolytic function of MutLalpha in human mismatch repair. *Cell* **126**: 297–308.

Kallastu, A., Hõrak, R., and Kivisaar, M. (1998) Identification and characterization of IS*1411*, a new insertion sequence which causes transcriptional activation of the phenol degradation genes in *Pseudomonas putida*. *J Bacteriol* **180**: 5306–5312.

Kamiya, H., and Kasai, H. (1995) Formation of 2-hydroxydeoxyadenosine triphosphate, an oxidatively damaged nucleotide, and its incorporation by DNA polymerases. Steady-state kinetics of the incorporation. *J Biol Chem* **270**: 19446–19450.

Kamiya, H., and Kasai, H. (1996) Effect of sequence contexts on misincorporation of nucleotides opposite 2-hydroxyadenine. *FEBS Lett* **391**: 113–116.

Kamiya, H., and Kasai, H. (1997) Substitution and deletion mutations induced by 2-hydroxyadenine in *Escherichia coli:* effects of sequence contexts in leading and lagging strands. *Nucleic Acids Res* **25**: 304–311.

Kamiya, H., and Kasai, H. (2000) 2-Hydroxy-dATP is incorporated opposite G by *Escherichia coli* DNA polymerase III resulting in high mutagenicity. *Nucleic Acids Res.* **28**: 1640–1646.

26

Kamiya, H., Murata-Kamiya, N., Iida, E., and Harashima, H. (2001) Hydrolysis of oxidized nucleotides by the *Escherichia coli* Orf135 protein. *Biochem. Biophys. Res. Commun.* **288**: 499–502.

Kamiya, H. (2003) Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/nitrogen species: approaches using synthetic oligonucleotides and nucleotides: survey and summary. *Nucleic Acids Res* **31**: 517–531.

Kamiya, H., Iida, E., Murata-Kamiya, N., Yamamoto, Y., Miki, T., and Harashima, H. (2003) Suppression of spontaneous and hydrogen peroxide-induced mutations by a MutT-type nucleotide pool sanitization enzyme, the *Escherichia coli* Orf135 protein. *Genes Cells.* **8**: 941–950.

Kamiya, H. (2004) Mutagenicities of 8-hydroxyguanine and 2-hydroxyadenine produced by reactive oxygen species. *Biol Pharm Bull* **27**: 475–479.

Kamiya, H., Ishiguro, C., and Harashima, H. (2004) Increased A:T-->C:G mutations in the *mutT* strain upon 8-hydroxy-dGTP treatment: direct evidence for MutT involvement in the prevention of mutations by oxidized dGTP. *J Biochem (Tokyo).* **136**: 359–362.

Kang, L.W., Gabelli, S.B., Bianchet, M.A., Xu, W.L., Bessman, M.J., and Amzel, L.M. (2003) Structure of a coenzyme A pyrophosphatase from Deinococcus radiodurans: a member of the Nudix family. *J Bacteriol* **185**: 4110–4118.

Kasak, L., Hõrak, R., and Kivisaar, M. (1997) Promoter-creating mutations in *Pseudomonas putida*: a model system for the study of mutation in starving bacteria. *Proc Natl Acad Sci USA* **94**: 3134–3139.

Kataoka, H., Yamamoto, Y., and Sekiguchi, M. (1983) A new gene (*alkB*) of *Escherichia coli* that controls sensitivity to methyl methane sulfonate. *J Bacteriol* **153**: 1301–1307.

Kauko, A., Haataja, S., Pulliainen, A.T., Finne, J., and Papageorgiou, A.C. (2004) Crystal structure of *Streptococcus suis* Dps-like peroxide resistance protein Dpr: implications for iron incorporation. *J Mol Biol* **338**: 547–558.

Kelman, Z., and Hurwitz, J. (1998) Protein-PCNA interactions: a DNA-scanning mechanism? *Trends Biochem Sci* **23**: 236–238.

Kelner, A. (1949) Photoreactivation of Ultraviolet-Irradiated *Escherichia coli*, with Special Reference to the Dose-Reduction Principle and to Ultraviolet-Induced Mutation. *J Bacteriol* **58**: 511–522.

Keyer, K., and Imlay, J.A. (1996) Superoxide accelerates DNA damage by elevating free-iron levels. *Proc Natl Acad Sci USA* **93**: 13635–13640.

Keyer, K., and Imlay, J.A. (1997) Inactivation of dehydratase [4Fe-4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. *J Biol Chem* **272**: 27652–27659.

Kim, S.R., Maenhaut-Michel, G., Yamada, M., Yamamoto, Y., Matsui, K., Sofuni, T., Nohmi, T., and Ohmori, H. (1997) Multiple pathways for SOS-induced mutagenesis in *Escherichia coli*: an overexpression of *dinB/dinP* results in strongly enhancing mutagenesis in the absence of any exogenous treatment to damage DNA. *Proc Natl Acad Sci USA.* **94**: 13792–13797.

Kim, S.R., Matsui, K., Yamada, M., Gruz, P., and Nohmi, T. (2001) Roles of chromosomal and episomal *dinB* genes encoding DNA pol IV in targeted and untargeted mutagenesis in *Escherichia coli*. *Mol Genet Genomics* **266**: 207–215.

Kino, K., and Sugiyama, H. (2001) Possible cause of G-C-->C-G transversion mutation by guanine oxidation product, imidazolone. *Chem Biol* **8**: 369–378.

Kivisaar, M., Kasak, L., and Nurk, A. (1991) Sequence of the plasmid-encoded catechol 1,2-dioxygenase-expressing gene, *pheB*, of phenol-degrading *Pseudomonas* sp. strain EST1001. *Gene* **98**: 15–20.

Kivisaar, M. (2003) Stationary phase mutagenesis: mechanisms that accelerate adaptation of microbial populations under environmental stress. *Environ Microbiol* **5**: 814–827.

Klein, J.C., Bleeker, M.J., Saris, C.P., Roelen, H.C., Brugghe, H.F., van den Elst, H., van der Marel, G.A., van Boom, J.H., Westra, J.G., Kriek, E., and et al. (1992) Repair and replication of plasmids with site-specific 8-oxodG and 8-AAFdG residues in normal and repair-deficient human cells. *Nucleic Acids Res* **20**: 4437–4443.

Kobayashi, M., Ohara-Nemoto, Y., Kaneko, M., Hayakawa, H., Sekiguchi, M., and Yamamoto, K. (1998) Potential of *Escherichia coli* GTP cyclohydrolase II for hydrolyzing 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. *J Biol Chem.* **273**: 26394–26399.

Kokubo, K., Yamada, M., Kanke, Y., and Nohmi, T. (2005) Roles of replicative and specialized DNA polymerases in frameshift mutagenesis: mutability of *Salmonella typhimurium* strains lacking one or all of SOS-inducible DNA polymerases to 26 chemicals. *DNA Repair (Amst)* **4**: 1160–1171.

Kolodner, R. (1996) Biochemistry and genetics of eukaryotic mismatch repair. *Genes Dev* **10**: 1433–1442.

Kolodner, R.D., and Marsischky, G.T. (1999) Eukaryotic DNA mismatch repair. *Curr Opin Genet Dev* **9**: 89–96.

Kolter, R., Siegele, D.A., and Tormo, A. (1993) The stationary phase of the bacterial life cycle. *Annu Rev Microbiol* **47**: 855–874.

Koonin, E.V. (1993) A highly conserved sequence motif defining the family of MutT-related proteins from eubacteria, eukaryotes and viruses. *Nucleic Acids Res* **21**: 4847.

Koorits, L., Tegova, R., Tark, M., Tarassova, K., Tover, A., and Kivisaar, M. (2007) Study of involvement of ImuB and DnaE2 in stationary-phase mutagenesis in *Pseudomonas putida*. *DNA Repair (Amst)* **6**: 863–868.

Kornyushyna, O., Berges, A.M., Muller, J.G., and Burrows, C.J. (2002) In vitro nucleotide misinsertion opposite the oxidized guanosine lesions spiroimino-dihydantoin and guanidinohydantoin and DNA synthesis past the lesions using *Escherichia coli* DNA polymerase I (Klenow fragment). *Biochemistry* **41**: 15304–15314.

Kornyushyna, O., and Burrows, C.J. (2003) Effect of the oxidized guanosine lesions spiroiminodihydantoin and guanidinohydantoin on proofreading by *Escherichia coli* DNA polymerase I (Klenow fragment) in different sequence contexts. *Biochemistry* **42**: 13008–13018.

Kotewicz, M.L., Brown, E.W., Eugene LeClerc, J., and Cebula, T.A. (2003) Genomic variability among enteric pathogens: the case of the *mutS-rpoS* intergenic region. *Trends Microbiol.* **11**: 2–6.

Kovtun, I.V., and McMurray, C.T. (2007) Crosstalk of DNA glycosylases with pathways other than base excision repair. *DNA Repair (Amst)* **6**: 517–529.

Kreuzer, K.N. (2005) Interplay between DNA replication and recombination in prokaryotes. *Annu Rev Microbiol* **59**: 43–67.

Krokan, H.E., Standal, R., and Slupphaug, G. (1997) DNA glycosylases in the base excision repair of DNA. *Biochem J* **325:** 1–16.

Krwawicz, J., Arczewska, K.D., Speina, E., Maciejewska, A., and Grzesiuk, E. (2007) Bacterial DNA repair genes and their eukaryotic homologues: 1. Mutations in genes involved in base excision repair (BER) and DNA-end processors and their implication in mutagenesis and human disease. *Acta Biochim Pol* **54**: 413-434

Kuban, W., Jonczyk, P., Gawel, D., Malanowska, K., Schaaper, R.M., and Fijalkowska, I.J. (2004) Role of *Escherichia coli* DNA polymerase IV in in vivo replication fidelity. *J Bacteriol* **186**: 4802–4807.

Kunkel, T.A., and Erie, D.A. (2005) DNA mismatch repair. *Annu Rev Biochem* **74**: 681–710.

Kuo, C.F., McRee, D.E., Fisher, C.L., O'Handley, S.F., Cunningham, R.P., and Tainer, J.A. (1992) Atomic structure of the DNA repair [4Fe-4S] enzyme endonuclease III. *Science* **258**: 434–440.

Kurtz, D.M., Jr. (2004) Microbial detoxification of superoxide: the non-heme iron reductive paradigm for combating oxidative stress. *Acc Chem Res* **37**: 902–908.

Kurusu, Y., Narita, T., Suzuki, M., and Watanabe, T. (2000) Genetic analysis of an incomplete *mutS* gene from *Pseudomonas putida*. *J Bacteriol* **182**: 5278–5279.

Lacks, S.A., Dunn, J.J., and Greenberg, B. (1982) Identification of base mismatches recognized by the heteroduplex-DNA-repair system of *Streptococcus pneumoniae*. *Cell* **31**: 327–336.

Lahue, R.S., Au, K.G., and Modrich, P. (1989) DNA mismatch correction in a defined system. *Science* **245**: 160–164.

Lamers, M.H., Perrakis, A., Enzlin, J.H., Winterwerp, H.H., de Wind, N., and Sixma, T.K. (2000) The crystal structure of DNA mismatch repair protein MutS binding to a G x T mismatch. *Nature.* **407**: 711–717.

Layton, J.C., and Foster, P.L. (2003) Error-prone DNA polymerase IV is controlled by the stress-response sigma factor, RpoS, in *Escherichia coli*. *Mol Microbiol.* **50**: 549–561.

LeClerc, J.E., Li, B., Payne, W.L., and Cebula, T.A. (1996) High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. *Science* **274**: 1208–1211.

Leipold, M.D., Muller, J.G., Burrows, C.J., and David, S.S. (2000) Removal of hydantoin products of 8-oxoguanine oxidation by the *Escherichia coli* DNA repair enzyme, FPG. *Biochemistry* **39**: 14984–14992.

Li, B., Tsui, H.C., LeClerc, J.E., Dey, M., Winkler, M.E., and Cebula, T.A. (2003) Molecular analysis of *mutS* expression and mutation in natural isolates of pathogenic *Escherichia coli*. *Microbiology* **149**: 1323–1331.

Li, X., Wright, P.M., and Lu, A.L. (2000) The C-terminal domain of MutY glycosylase determines the 7,8-dihydro-8-oxo-guanine specificity and is crucial for mutation avoidance. *J Biol Chem.* **275**: 8448–8455.

Lin, J., Abeygunawardana, C., Frick, D.N., Bessman, M.J., and Mildvan, A.S. (1997) Solution structure of the quaternary MutT-M2+-AMPCPP-M2+ complex and mechanism of its pyrophosphohydrolase action. *Biochemistry* **36**: 1199–1211.

Lin, J.J., and Sancar, A. (1989) A new mechanism for repairing oxidative damage to DNA: (A)BC excinuclease removes AP sites and thymine glycols from DNA. *Biochemistry* **28**: 7979–7984.

Lindahl, T. (1974) An *N*-glycosidase from *Escherichia coli* that releases free uracil from DNA containing deaminated cytosine residues. *Proc Natl Acad Sci USA* **71**: 3649–3653.

Lindahl, T., Sedgwick, B., Sekiguchi, M., and Nakabeppu, Y. (1988) Regulation and expression of the adaptive response to alkylating agents. *Annu Rev Biochem* **57**: 133–157.

Lindahl, T. (1993) Instability and decay of the primary structure of DNA. *Nature* **362**: 709–715.

Ling, H., Boudsocq, F., Woodgate, R., and Yang, W. (2001) Crystal structure of a Y-family DNA polymerase in action: a mechanism for error-prone and lesion-bypass replication. *Cell* **107**: 91–102.

Liochev, S.I., and Fridovich, I. (1994) The role of O2.- in the production of HO.: in vitro and in vivo. *Free Radic Biol Med* **16**: 29–33.

Loewe, L., Textor, V., and Scherer, S. (2003) High deleterious genomic mutation rate in stationary phase of *Escherichia coli*. *Science* **302**: 1558–1560.

Loewen, P.C., and Hengge-Aronis, R. (1994) The role of the sigma factor sigma S (KatF) in bacterial global regulation. *Annu Rev Microbiol* **48**: 53–80.

Lombardo, M.J., Aponyi, I., and Rosenberg, S.M. (2004) General stress response regulator RpoS in adaptive mutation and amplification in *Escherichia coli*. *Genetics* **166**: 669–680.

Longerich, S., Galloway, A.M., Harris, R.S., Wong, C., and Rosenberg, S.M. (1995) Adaptive mutation sequences reproduced by mismatch repair deficiency. *Proc Natl Acad Sci USA* **92**: 12017–12020.

Lopes, M., Foiani, M., and Sogo, J.M. (2006) Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions. *Mol Cell* **21**: 15–27.

Lopez de Saro, F.J., and O'Donnell, M. (2001) Interaction of the beta sliding clamp with MutS, ligase, and DNA polymerase I. *Proc Natl Acad Sci USA* **98**: 8376–8380.

Lopez de Saro, F.J., Georgescu, R.E., Goodman, M.F., and O'Donnell, M. (2003) Competitive processivity-clamp usage by DNA polymerases during DNA replication and repair. *EMBO J* **22**: 6408–6418.

Lopez de Saro, F.J., Marinus, M.G., Modrich, P., and O'Donnell, M. (2006) The beta sliding clamp binds to multiple sites within MutL and MutS. *J Biol Chem* **281**: 14340–14349.

Lu, A.L., Clark, S., and Modrich, P. (1983) Methyl-directed repair of DNA base-pair mismatches in vitro. *Proc Natl Acad Sci USA* **80**: 4639–4643.

Lu, A.L., and Chang, D.Y. (1988) Repair of single base-pair transversion mismatches of *Escherichia coli in vitro*: correction of certain A/G mismatches is independent of *dam* methylation and host *mutHLS* gene functions. *Genetics* **118**: 593–600.

Lu, A.L., Lee, C.Y., Li, L., and Li, X. (2006) Physical and functional interactions between Escherichia coli MutY and endonuclease VIII. *Biochem J.* **393**: 381–387.

Luo, W., Muller, J.G., Rachlin, E.M., and Burrows, C.J. (2000) Characterization of spiroiminodihydantoin as a product of one-electron oxidation of 8-Oxo-7,8-dihydroguanosine. *Org Lett* **2**: 613–616.

27

Luo, W., Muller, J.G., Rachlin, E.M., and Burrows, C.J. (2001) Characterization of hydantoin products from one-electron oxidation of 8-oxo-7,8-dihydroguanosine in a nucleoside model. *Chem Res Toxicol* **14**: 927–938.

Luria, S.A., and Delbrück, M. (1943) Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28**: 491–511.

Maki, H., and Sekiguchi, M. (1992) MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature.* **355**: 273–275.

Malik, H.S., and Henikoff, S. (2000) Dual recognition-incision enzymes might be involved in mismatch repair and meiosis. *Trends Biochem Sci* **25**: 414–418.

Manuel, R.C., Czerwinski, E.W., and Lloyd, R.S. (1996) Identification of the structural and functional domains of MutY, an *Escherichia coli* DNA mismatch repair enzyme. *J Biol Chem* **271**: 16218–16226.

Manuel, R.C., and Lloyd, R.S. (1997) Cloning, overexpression, and biochemical characterization of the catalytic domain of MutY. *Biochemistry* **36**: 11140–11152.

Marti, T.M., Kunz, C., and Fleck, O. (2002) DNA mismatch repair and mutation avoidance pathways. *J Cell Physiol.* **191**: 28–41.

Martinez, A., and Kolter, R. (1997) Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. *J Bacteriol* **179**: 5188–5194.

Massey, V. (1994) Activation of molecular oxygen by flavins and flavoproteins. *J Biol Chem* **269**: 22459–22462.

Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K., and Hanaoka, F. (1999) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. *Nature* **399**: 700–704.

Mazurek, A., Berardini, M., and Fishel, R. (2002) Activation of human MutS homologs by 8-oxo-guanine DNA damage. *J Biol Chem* **277**: 8260–8266.

Matic, I., Taddei, F., and Radman, M. (1996) Genetic barriers among bacteria. *Trends Microbiol* **4**: 69–72.

Matic, I., Radman, M., Taddei, F., Picard, B., Doit, C., Bingen, E., Denamur, E., and Elion, J. (1997) Highly variable mutation rates in commensal and pathogenic *Escherichia coli*. *Science* **277**: 1833–1834.

Matic, I., Babic, A., and Radman, M. (2003) 2-aminopurine allows interspecies recombination by a reversible inactivation of the *Escherichia coli* mismatch repair system. *J Bacteriol* **185**: 1459–1461.

Matsumoto, Y., Zhang, Q.M., Takao, M., Yasui, A., and Yonei, S. (2001) *Escherichia coli* Nth and human hNTH1 DNA glycosylases are involved in removal of 8-oxoguanine from 8-oxoguanine/guanine mispairs in DNA. *Nucleic Acids Res* **29**: 1975–1981.

McGlynn, P., and Lloyd, R.G. (2002) Recombinational repair and restart of damaged replication forks. *Nat Rev Mol Cell Biol* **3**: 859–870.

McKenzie, G.J., Harris, R.S., Lee, P.L., and Rosenberg, S.M. (2000) The SOS response regulates adaptive mutation. *Proc Natl Acad Sci USA*. **97**: 6646–6651.

McKenzie, G.J., Lee, P.L., Lombardo, M.J., Hastings, P.J., and Rosenberg, S.M. (2001) SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. *Mol Cell.* **7**: 571–579.

McLennan, A.G. (2006) The Nudix hydrolase superfamily. *Cell Mol Life Sci* **63**: 123–143.

Mechanic, L.E., Frankel, B.A., and Matson, S.W. (2000) *Escherichia coli* MutL loads DNA helicase II onto DNA. *J Biol Chem* **275**: 38337–38346.
Meier, P., and Wackernagel, W. (2005) Impact of *mutS* inactivation on foreign DNA acquisition by natural transformation in *Pseudomonas stutzeri*. *J Bacteriol* **187**: 143–154.
Melamede, R.J., Hatahet, Z., Kow, Y.W., Ide, H., and Wallace, S.S. (1994) Isolation and characterization of endonuclease VIII from *Escherichia coli*. *Biochemistry* **33**: 1255–1264.
Michaels, M.L., Pham, L., Nghiem, Y., Cruz, C., and Miller, J.H. (1990) MutY, an adenine glycosylase active on G-A mispairs, has homology to endonuclease III. *Nucleic Acids Res* **18**: 3841–3845.
Michaels, M.L., Pham, L., Cruz, C., and Miller, J.H. (1991) MutM, a protein that prevents G.C----T.A transversions, is formamidopyrimidine-DNA glycosylase. *Nucleic Acids Res* **19**: 3629–3632.
Michaels, M.L., Cruz, C., Grollman, A.P., and Miller, J.H. (1992a) Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. *Proc Natl Acad Sci USA*. **89**: 7022–7025.
Michaels, M.L., and Miller, J.H. (1992) The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). *J Bacteriol*. **174**: 6321–6325.
Michaels, M.L., Tchou, J., Grollman, A.P., and Miller, J.H. (1992b) A repair system for 8-oxo-7,8-dihydrodeoxyguanine. *Biochemistry*. **31**: 10964–10968.
Mildvan, A.S., Xia, Z., Azurmendi, H.F., Saraswat, V., Legler, P.M., Massiah, M.A., Gabelli, S.B., Bianchet, M.A., Kang, L.W., and Amzel, L.M. (2005) Structures and mechanisms of Nudix hydrolases. *Arch Biochem Biophys* **433**: 129–143.
Miller, J.H. (1996) Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. *Annu Rev Microbiol* **50**: 625–643.
Miller, J.H. (1998) Mutators in *Escherichia coli*. *Mutat Res* **409**: 99–106.
Miller, J.H., Suthar, A., Tai, J., Yeung, A., Truong, C., and Stewart, J.L. (1999) Direct selection for mutators in *Escherichia coli*. *J Bacteriol*. **181**: 1576–1584.
Minsky, A., Shimoni, E., and Frenkiel-Krispin, D. (2002) Stress, order and survival. *Nat Rev Mol Cell Biol* **3**: 50–60.
Mitra, S., Boldogh, I., Izumi, T., and Hazra, T.K. (2001) Complexities of the DNA base excision repair pathway for repair of oxidative DNA damage. *Environ Mol Mutagen* **38**: 180–190.
Mo, J.Y., Maki, H., and Sekiguchi, M. (1992) Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool. *Proc Natl Acad Sci USA* **89**: 11021–11025.
Modrich, P. (1991) Mechanisms and biological effects of mismatch repair. *Annu Rev Genet* **25**: 229–253.
Modrich, P., and Lahue, R. (1996) Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu Rev Biochem* **65**: 101–133.
Modrich, P. (1997) Strand-specific mismatch repair in mammalian cells. *J Biol Chem* **272**: 24727–24730.
Modrich, P. (2006) Mechanisms in eukaryotic mismatch repair. *J Biol Chem* **11**: 11.
Moeller, R., Stackebrandt, E., Reitz, G., Berger, T., Rettberg, P., Doherty, A.J., Horneck, G., and Nicholson, W.L. (2007) Role of DNA repair by nonhomologous-

end joining in *Bacillus subtilis* spore resistance to extreme dryness, mono- and polychromatic UV, and ionizing radiation. *J Bacteriol* **189**: 3306–3311.

Mol, C.D., Kuo, C.F., Thayer, M.M., Cunningham, R.P., and Tainer, J.A. (1995) Structure and function of the multifunctional DNA-repair enzyme exonuclease III. *Nature* **374**: 381–386.

Mol, C.D., Hosfield, D.J., and Tainer, J.A. (2000) Abasic site recognition by two apurinic/apyrimidinic endonuclease families in DNA base excision repair: the 3' ends justify the means. *Mutat Res* **460**: 211–229.

Moriya, M., Ou, C., Bodepudi, V., Johnson, F., Takeshita, M., and Grollman, A.P. (1991) Site-specific mutagenesis using a gapped duplex vector: a study of translesion synthesis past 8-oxodeoxyguanosine in *E. coli*. *Mutat Res* **254**: 281–288.

Moriya, M., and Grollman, A.P. (1993) Mutations in the *mutY* gene of *Escherichia coli* enhance the frequency of targeted G:C-->T:A transversions induced by a single 8-oxoguanine residue in single-stranded DNA. *Mol Gen Genet* **239**: 72–76.

Msadek, T. (1999) When the going gets tough: survival strategies and environmental signaling networks in *Bacillus subtilis*. *Trends Microbiol* **7**: 201–207.

Nair, S., and Finkel, S.E. (2004) Dps protects cells against multiple stresses during stationary phase. *J Bacteriol* **186**: 4192–4198.

Natrajan, G., Lamers, M.H., Enzlin, J.H., Winterwerp, H.H., Perrakis, A., and Sixma, T.K. (2003) Structures of *Escherichia coli* DNA mismatch repair enzyme MutS in complex with different mismatches: a common recognition mode for diverse substrates. *Nucleic Acids Res* **31**: 4814–4821.

Neeley, W.L., Delaney, J.C., Henderson, P.T., and Essigmann, J.M. (2004) In vivo bypass efficiencies and mutational signatures of the guanine oxidation products 2-aminoimidazolone and 5-guanidino-4-nitroimidazole. *J Biol Chem* **279**: 43568–43573.

Neeley, W.L., and Essigmann, J.M. (2006) Mechanisms of formation, genotoxicity, and mutation of guanine oxidation products. *Chem Res Toxicol* **19**: 491–505.

Neeley, W.L., Delaney, S., Alekseyev, Y.O., Jarosz, D.F., Delaney, J.C., Walker, G.C., and Essigmann, J.M. (2007) DNA polymerase V allows bypass of toxic guanine oxidation products *in vivo*. *J Biol Chem* **282**: 12741–12748.

Negishi, K., Loakes, D., and Schaaper, R.M. (2002) Saturation of DNA mismatch repair and error catastrophe by a base analogue in *Escherichia coli*. *Genetics* **161**: 1363–1371.

Newcombe, H.B., and Whitehead, H.A. (1951) Photoreversal of ultraviolet-induced mutagenic and lethal effects in *Escherichia coli*. *J Bacteriol* **61**: 243–251.

Nghiem, Y., Cabrera, M., Cupples, C.G., and Miller, J.H. (1988) The *mutY* gene: a mutator locus in *Escherichia coli* that generates G.C----T.A transversions. *Proc Natl Acad Sci USA* **85**: 2709–2713.

Nieminuszczy, J., and Grzesiuk, E. (2007) Bacterial DNA repair genes and their eukaryotic homologues: 3 AlkB dioxygenase and Ada methyltransferase in the direct repair of alkylated DNA. *Acta Biochim Pol* **54**: 459-468

Niles, J.C., Wishnok, J.S., and Tannenbaum, S.R. (2001a) Spiroiminodihydantoin is the major product of the 8-oxo-7,8-dihydroguanosine reaction with peroxynitrite in the presence of thiols and guanosine photooxidation by methylene blue. *Org Lett* **3**: 963–966.

Niles, J.C., Wishnok, J.S., and Tannenbaum, S.R. (2001b) A novel nitroimidazole compound formed during the reaction of peroxynitrite with 2',3',5'-tri-O-acetyl-guanosine. *J Am Chem Soc* **123**: 12147–12151.

Nohmi, T. (2006) Environmental stress and lesion-bypass DNA polymerases. *Annu Rev Microbiol* **60**: 231–253.

Noll, D.M., Gogos, A., Granek, J.A., and Clarke, N.D. (1999) The C-terminal domain of the adenine-DNA glycosylase MutY confers specificity for 8-oxoguanine.adenine mispairs and may have evolved from MutT, an 8-oxo-dGTPase. *Biochemistry* **38**: 6374–6379.

Nurk, A., Kasak, L., and Kivisaar, M. (1991) Sequence of the gene (*pheA*) encoding phenol monooxygenase from *Pseudomonas* sp. EST1001: expression in *Escherichia coli* and *Pseudomonas putida*. *Gene* **102**: 13–18.

Nurk, A., Tamm, A., Horak, R., and Kivisaar, M. (1993) In-vivo-generated fusion promoters in *Pseudomonas putida*. *Gene* **127**: 23–29.

Nyström, T. (2004) Stationary-phase physiology. *Annu Rev Microbiol* **58**: 161–181.

O'Handley, S.F., Dunn, C.A., and Bessman, M.J. (2001) Orf135 from *Escherichia coli* Is a Nudix hydrolase specific for CTP, dCTP, and 5-methyl-dCTP. *J Biol Chem* **276**: 5421–5426.

Obmolova, G., Ban, C., Hsieh, P., and Yang, W. (2000) Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. *Nature.* **407**: 703–710.

Ojangu, E.L., Tover, A., Teras, R., and Kivisaar, M. (2000) Effects of combination of different –10 hexamers and downstream sequences on stationary-phase-specific sigma factor sigma(S)-dependent transcription in *Pseudomonas putida*. *J Bacteriol* **182**: 6707–6713.

Oliver, A., Canton, R., Campo, P., Baquero, F., and Blazquez, J. (2000) High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* **288**: 1251–1254.

Oliver, A., Baquero, F., and Blazquez, J. (2002a) The mismatch repair system (*mutS*, *mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Mol Microbiol* **43**: 1641–1650.

Oliver, A., Sanchez, J.M., and Blazquez, J. (2002b) Characterization of the GO system of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **217**: 31–35.

Ono, T., Negishi, K., and Hayatsu, H. (1995) Spectra of superoxide-induced mutations in the *lacI* gene of a wild-type and a *mutM* strain of *Escherichia coli* K-12. *Mutat Res* **326**: 175–183.

Papinutto, E., Dundon, W.G., Pitulis, N., Battistutta, R., Montecucco, C., and Zanotti, G. (2002) Structure of two iron-binding proteins from *Bacillus anthracis*. *J Biol Chem* **277**: 15093–15098.

Parikh, S.S., Mol, C.D., and Tainer, J.A. (1997) Base excision repair enzyme family portrait: integrating the structure and chemistry of an entire DNA repair pathway. *Structure* **5**: 1543–1550.

Pedraza-Reyes, M., and Yasbin, R.E. (2004) Contribution of the mismatch DNA repair system to the generation of stationary-phase-induced mutants of *Bacillus subtilis*. *J Bacteriol* **186**: 6485–6491.

Pitcher, R.S., Brissett, N.C., and Doherty, A.J. (2007a) Nonhomologous End-Joining in Bacteria: A Microbial Perspective. *Annu Rev Microbiol* **61**: 259–282.

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Pitcher, R.S., Green, A.J., Brzostek, A., Korycka-Machala, M., Dziadek, J., and Doherty, A.J. (2007b) NHEJ protects mycobacteria in stationary phase against the harmful effects of desiccation. *DNA Repair (Amst)* **6**: 1271-1276

Potter, P.M., Wilkinson, M.C., Fitton, J., Carr, F.J., Brennand, J., Cooper, D.P., and Margison, G.P. (1987) Characterisation and nucleotide sequence of *ogt*, the O6-alkylguanine-DNA-alkyltransferase gene of *E. coli*. *Nucleic Acids Res* **15**: 9177–9193.

Prival, M.J., and Cebula, T.A. (1992) Sequence analysis of mutations arising during prolonged starvation of *Salmonella typhimurium*. *Genetics* **132**: 303–310.

Purmal, A.A., Lampman, G.W., Bond, J.P., Hatahet, Z., and Wallace, S.S. (1998) Enzymatic processing of uracil glycol, a major oxidative product of DNA cytosine. *J Biol Chem* **273**: 10026–10035.

Qiu, Z., and Goodman, M.F. (1997) The *Escherichia coli polB* locus is identical to *dinA*, the structural gene for DNA polymerase II. Characterization of Pol II purified from a *polB* mutant. *J Biol Chem* **272**: 8611–8617.

Radicella, J.P., Clark, E.A., and Fox, M.S. (1988) Some mismatch repair activities in *Escherichia coli*. *Proc Natl Acad Sci USA* **85**: 9674–9678.

Radicella, J.P., Park, P.U., and Fox, M.S. (1995) Adaptive mutation in *Escherichia coli*: a role for conjugation. *Science* **268**: 418–420.

Rainey, B.P., and Moxon, E.R. (2000) Microbiology. When being hyper keeps you fit. *Science* **288**: 1186–1187.

Ramsay, B., Wiedenheft, B., Allen, M., Gauss, G.H., Lawrence, C.M., Young, M., and Douglas, T. (2006) Dps-like protein from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Inorg Biochem* **100**: 1061–1068.

Rattray, A.J., and Strathern, J.N. (2003) Error-prone DNA polymerases: when making a mistake is the only way to get ahead. *Annu Rev Genet* **37**: 31–66.

Rayssiguier, C., Thaler, D.S., and Radman, M. (1989) The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants. *Nature* **342**: 396–401.

Reardon, J.T., and Sancar, A. (2005) Nucleotide excision repair. *Prog Nucleic Acid Res Mol Biol* **79**: 183–235.

Reindel, S., Anemuller, S., Sawaryn, A., and Matzanke, B.F. (2002) The DpsA-homologue of the archaeon *Halobacterium salinarum* is a ferritin. *Biochim Biophys Acta* **1598**: 140–146.

Reuven, N.B., Arad, G., Maor-Shoshani, A., and Livneh, Z. (1999) The mutagenesis protein UmuC is a DNA polymerase activated by UmuD', RecA, and SSB and is specialized for translesion replication. *J Biol Chem* **274**: 31763–31766.

Richardson, A.R., Yu, Z., Popovic, T., and Stojiljkovic, I. (2002) Mutator clones of *Neisseria meningitidis* in epidemic serogroup A disease. *Proc Natl Acad Sci USA* **99**: 6103–6107.

Rosche, W.A., and Foster, P.L. (1999) The role of transient hypermutators in adaptive mutation in *Escherichia coli*. *Proc Natl Acad Sci USA*. **96**: 6862–6867.

Rosenberg, S.M., Longerich, S., Gee, P., and Harris, R.S. (1994) Adaptive mutation by deletions in small mononucleotide repeats. *Science* **265**: 405–407.

Rosenberg, S.M., Thulin, C., and Harris, R.S. (1998) Transient and heritable mutators in adaptive evolution in the lab and in nature. *Genetics* **148**: 1559–1566.

Rosenberg, S.M. (2001) Evolving responsively: adaptive mutation. *Nat Rev Genet* **2**: 504–515.

Ross, C., Pybus, C., Pedraza-Reyes, M., Sung, H.M., Yasbin, R.E., and Robleto, E. (2006) Novel role of *mfd*: effects on stationary-phase mutagenesis in *Bacillus subtilis*. *J Bacteriol* **188**: 7512–7520.

Roth, J.R., and Andersson, D.I. (2004) Adaptive mutation: how growth under selection stimulates Lac(+) reversion by increasing target copy number. *J Bacteriol* **186**: 4855–4860.

Roth, J.R., Kugelberg, E., Reams, A.B., Kofoid, E., and Andersson, D.I. (2006) Origin of mutations under selection: the adaptive mutation controversy. *Annu Rev Microbiol* **60**: 477–501.

Roy, S., Gupta, S., Das, S., Sekar, K., Chatterji, D., and Vijayan, M. (2004) X-ray analysis of *Mycobacterium smegmatis* Dps and a comparative study involving other Dps and Dps-like molecules. *J Mol Biol* **339**: 1103–1113.

Russo, M.T., Blasi, M.F., Chiera, F., Fortini, P., Degan, P., Macpherson, P., Furuichi, M., Nakabeppu, Y., Karran, P., Aquilina, G., and Bignami, M. (2004) The oxidized deoxynucleoside triphosphate pool is a significant contributor to genetic instability in mismatch repair-deficient cells. *Mol Cell Biol* **24**: 465–474.

Ryan, F.J., Nakada, D., and Schneider, M.J. (1961) Is DNA replication a necessary condition for spontaneous mutation? *Z Vererbungsl* **92**: 38–41.

Saito, Y., Uraki, F., Nakajima, S., Asaeda, A., Ono, K., Kubo, K., and Yamamoto, K. (1997) Characterization of endonuclease III (nth) and endonuclease VIII (nei) mutants of Escherichia coli K-12. *J Bacteriol* **179**: 3783–3785.

Sakumi, K., Furuichi, M., Tsuzuki, T., Kakuma, T., Kawabata, S., Maki, H., and Sekiguchi, M. (1993) Cloning and expression of cDNA for a human enzyme that hydrolyzes 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. *J Biol Chem* **268**: 23524–23530.

Sancar, A., and Rupert, C.S. (1978) Cloning of the *phr* gene and amplification of photolyase in *Escherichia coli*. *Gene* **4**: 295–308.

Sancar, A., and Rupp, W.D. (1983) A novel repair enzyme: UVRABC excision nuclease of *Escherichia coli* cuts a DNA strand on both sides of the damaged region. *Cell* **33**: 249–260.

Sancar, A., and Hearst, J.E. (1993) Molecular matchmakers. *Science* **259**: 1415–1420.

Sancar, A. (1994) Structure and function of DNA photolyase. *Biochemistry* **33**: 2–9.

Sancar, A. (2003) Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. *Chem Rev* **103**: 2203–2237.

Sancar, A., and Reardon, J.T. (2004) Nucleotide excision repair in *E. coli* and man. *Adv Protein Chem* **69**: 43–71.

Sancar, G.B. (1990) DNA photolyases: physical properties, action mechanism, and roles in dark repair. *Mutat Res* **236**: 147–160.

Sandigursky, M., and Franklin, W.A. (1992) DNA deoxyribophosphodiesterase of *Escherichia coli* is associated with exonuclease I. *Nucleic Acids Res* **20**: 4699–4703.

Saparbaev, M., and Laval, J. (1994) Excision of hypoxanthine from DNA containing dIMP residues by the *Escherichia coli*, yeast, rat, and human alkylpurine DNA glycosylases. *Proc Natl Acad Sci USA* **91**: 5873–5877.

Saparbaev, M., and Laval, J. (1998) 3,N4-ethenocytosine, a highly mutagenic adduct, is a primary substrate for *Escherichia coli* double-stranded uracil-DNA glycosylase

and human mismatch-specific thymine-DNA glycosylase. *Proc Natl Acad Sci USA* **95**: 8508–8513.
Saparbaev, M., Langouet, S., Privezentzev, C.V., Guengerich, F.P., Cai, H., Elder, R.H., and Laval, J. (2002) 1,N(2)-ethenoguanine, a mutagenic DNA adduct, is a primary substrate of *Escherichia coli* mismatch-specific uracil-DNA glycosylase and human alkylpurine-DNA-N-glycosylase. *J Biol Chem* **277**: 26987–26993.
Satou, K., Yamada, M., Nohmi, T., Harashima, H., and Kamiya, H. (2005) Mutagenesis induced by oxidized DNA precursors: roles of Y family DNA polymerases in *Escherichia coli*. *Chem Res Toxicol* **18**: 1271–1278.
Schaaper, R.M., and Dunn, R.L. (1987) Spectra of spontaneous mutations in *Escherichia coli* strains defective in mismatch correction: the nature of in vivo DNA replication errors. *Proc Natl Acad Sci USA* **84**: 6220–6224.
Schaaper, R.M. (1988) Mechanisms of mutagenesis in the *Escherichia coli* mutator *mutD5*: role of DNA mismatch repair. *Proc Natl Acad Sci USA* **85**: 8126–8130.
Schaaper, R.M., and Radman, M. (1989) The extreme mutator effect of *Escherichia coli mutD*5 results from saturation of mismatch repair by excessive DNA replication errors. *EMBO J* **8**: 3511–3516.
Schaaper, R.M. (1993) Base selection, proofreading, and mismatch repair during DNA replication in *Escherichia coli*. *J Biol Chem* **268**: 23762–23765.
Schlacher, K., and Goodman, M.F. (2007) Lessons from 50 years of SOS DNA-damage-induced mutagenesis. *Nat Rev Mol Cell Biol* **8**: 587–594.
Schmid, M.B. (1990) More than just "histone-like" proteins. *Cell* **63**: 451–453.
Schofield, M.J., Nayak, S., Scott, T.H., Du, C., and Hsieh, P. (2001) Interaction of *Escherichia coli* MutS and MutL at a DNA mismatch. *J Biol Chem.* **276**: 28291–28299.
Schofield, M.J., and Hsieh, P. (2003) DNA mismatch repair: molecular mechanisms and biological function. *Annu Rev Microbiol* **57**: 579–608.
Sedgwick, B., Bates, P.A., Paik, J., Jacobs, S.C., and Lindahl, T. (2007) Repair of alkylated DNA: recent advances. *DNA Repair (Amst)* **6**: 429–442.
Seeberg, E., Eide, L., and Bjoras, M. (1995) The base excision repair pathway. *Trends Biochem Sci* **20**: 391–397.
Selmane, T., Schofield, M.J., Nayak, S., Du, C., and Hsieh, P. (2003) Formation of a DNA mismatch repair complex mediated by ATP. *J Mol Biol* **334**: 949–965.
Serre, L., Pereira de Jesus, K., Boiteux, S., Zelwer, C., and Castaing, B. (2002) Crystal structure of the *Lactococcus lactis* formamidopyrimidine-DNA glycosylase bound to an abasic site analogue-containing DNA. *EMBO J* **21**: 2854–2865.
Shapiro, J.A. (2005) A 21st century view of evolution: genome system architecture, repetitive DNA, and natural genetic engineering. *Gene* **345**: 91–100.
Shibutani, S., Takeshita, M., and Grollman, A.P. (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* **349**: 431–434.
Shimizu, M., Gruz, P., Kamiya, H., Kim, S.R., Pisani, F.M., Masutani, C., Kanke, Y., Harashima, H., Hanaoka, F., and Nohmi, T. (2003) Erroneous incorporation of oxidized DNA precursors by Y-family DNA polymerases. *EMBO Rep.* **4**: 269–273.
Sixma, T.K. (2001) DNA mismatch repair: MutS structures bound to mismatches. *Curr Opin Struct Biol.* **11**: 47–52.

Slechta, E.S., Bunny, K.L., Kugelberg, E., Kofoid, E., Andersson, D.I., and Roth, J.R. (2003) Adaptive mutation: general mutagenesis is not a programmed response to stress but results from rare coamplification of *dinB* with *lac*. *Proc Natl Acad Sci USA* **100**: 12847–12852.

Slupphaug, G., Kavli, B., and Krokan, H.E. (2003) The interacting pathways for prevention and repair of oxidative DNA damage. *Mutat Res* **531**: 231–251.

Sniegowski, P.D., Gerrish, P.J., and Lenski, R.E. (1997) Evolution of high mutation rates in experimental populations of *E. coli*. *Nature* **387**: 703–705.

Spiers, A.J., Buckling, A., and Rainey, P.B. (2000) The causes of *Pseudomonas* diversity. *Microbiology* **146**: 2345–2350.

Stahl, F.W. (1988) Bacterial genetics. A unicorn in the garden. *Nature* **335**: 112–113.

Stambuk, S., and Radman, M. (1998) Mechanism and control of interspecies recombination in *Escherichia coli.* I. Mismatch repair, methylation, recombination and replication functions. *Genetics* **150**: 533–542.

Steinum, A.L., and Seeberg, E. (1986) Nucleotide sequence of the *tag* gene from *Escherichia coli*. *Nucleic Acids Res* **14**: 3763–3772.

Stephani, K., Weichart, D., and Hengge, R. (2003) Dynamic control of Dps protein levels by ClpXP and ClpAP proteases in *Escherichia coli*. *Mol Microbiol* **49**: 1605–1614.

Storz, G., and Imlay, J.A. (1999) Oxidative stress. *Curr Opin Microbiol* **2**: 188–194.

Strauss, B.S. (1991) The 'A rule' of mutagen specificity: a consequence of DNA polymerase bypass of non-instructional lesions? *Bioessays* **13**: 79–84.

Strauss, B.S., Roberts, R., Francis, L., and Pouryazdanparast, P. (2000) Role of the *dinB* gene product in spontaneous mutation in *Escherichia coli* with an impaired replicative polymerase. *J Bacteriol.* **182**: 6742–6750.

Stukenberg, P.T., Studwell-Vaughan, P.S., and O'Donnell, M. (1991) Mechanism of the sliding beta-clamp of DNA polymerase III holoenzyme. *J Biol Chem* **266**: 11328–11334.

Su, S.S., and Modrich, P. (1986) *Escherichia coli mutS*-encoded protein binds to mismatched DNA base pairs. *Proc. Natl. Acad. Sci. USA* **83**: 5057–5061.

Su, S.S., Lahue, R.S., Au, K.G., and Modrich, P. (1988) Mispair specificity of methyl-directed DNA mismatch correction in vitro. *J Biol Chem* **263**: 6829–6835.

Sugahara, M., Mikawa, T., Kumasaka, T., Yamamoto, M., Kato, R., Fukuyama, K., Inoue, Y., and Kuramitsu, S. (2000) Crystal structure of a repair enzyme of oxidatively damaged DNA, MutM (Fpg), from an extreme thermophile, *Thermus thermophilus* HB8. *EMBO J* **19**: 3857–3869.

Sun, B., Latham, K.A., Dodson, M.L., and Lloyd, R.S. (1995) Studies on the catalytic mechanism of five DNA glycosylases. Probing for enzyme-DNA imino intermediates. *J Biol Chem* **270**: 19501–19508.

Sundin, G.W., and Weigand, M.R. (2007) The microbiology of mutability. *FEMS Microbiol Lett*.

Sung, H.M., and Yasbin, R.E. (2002) Adaptive, or stationary-phase, mutagenesis, a component of bacterial differentiation in *Bacillus subtilis*. *J Bacteriol* **184**: 5641–5653.

Sung, H.M., Yeamans, G., Ross, C.A., and Yasbin, R.E. (2003) Roles of YqjH and YqjW, homologs of the *Escherichia coli* UmuC/DinB or Y superfamily of DNA

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polymerases, in stationary-phase mutagenesis and UV-induced mutagenesis of *Bacillus subtilis*. *J Bacteriol* **185**: 2153–2160.
Sung, J.S., and Demple, B. (2006) Roles of base excision repair subpathways in correcting oxidized abasic sites in DNA. *Febs J* **273**: 1620–1629.
Sutton, M.D., and Walker, G.C. (2001) Managing DNA polymerases: coordinating DNA replication, DNA repair, and DNA recombination. *Proc Natl Acad Sci USA*. **98**: 8342–8349.
Zambrano, M.M., Siegele, D.A., Almiron, M., Tormo, A., and Kolter, R. (1993) Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. *Science* **259**: 1757–1760.
Zanotti, G., Papinutto, E., Dundon, W., Battistutta, R., Seveso, M., Giudice, G., Rappuoli, R., and Montecucco, C. (2002) Structure of the neutrophil-activating protein from *Helicobacter pylori*. *J Mol Biol* **323**: 125–130.
Zeibell, K., Aguila, S., Yan Shi, V., Chan, A., Yang, H., and Miller, J.H. (2007) Mutagenesis and repair in *Bacillus anthracis*: the effect of mutators. *J Bacteriol* **189**: 2331–2338.
Zhang, Q.M., Ishikawa, N., Nakahara, T., and Yonei, S. (1998) *Escherichia coli* MutY protein has a guanine-DNA glycosylase that acts on 7,8-dihydro-8-oxoguanine:guanine mispair to prevent spontaneous G:C-->C:G transversions. *Nucleic Acids Res* **26**: 4669–4675.
Zhang, Q.M., Miyabe, I., Matsumoto, Y., Kino, K., Sugiyama, H., and Yonei, S. (2000) Identification of repair enzymes for 5-formyluracil in DNA. Nth, Nei, and MutM proteins of *Escherichia coli*. *J Biol Chem* **275**: 35471–35477.
Zhao, G., Ceci, P., Ilari, A., Giangiacomo, L., Laue, T.M., Chiancone, E., and Chasteen, N.D. (2002) Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. *J Biol Chem* **277**: 27689–27696.
Zhao, J., and Winkler, M.E. (2000) Reduction of GC --> TA transversion mutation by overexpression of MutS in *Escherichia coli* K-12. *J Bacteriol.* **182**: 5025–5028.
Zharkov, D.O., Golan, G., Gilboa, R., Fernandes, A.S., Gerchman, S.E., Kycia, J.H., Rieger, R.A., Grollman, A.P., and Shoham, G. (2002) Structural analysis of an *Escherichia coli* endonuclease VIII covalent reaction intermediate. *EMBO J* **21**: 789–800.
Taddei, F., Hayakawa, H., Bouton, M., Cirinesi, A., Matic, I., Sekiguchi, M., and Radman, M. (1997a) Counteraction by MutT protein of transcriptional errors caused by oxidative damage. *Science.* **278**: 128–130.
Taddei, F., Radman, M., Maynard-Smith, J., Toupance, B., Gouyon, P.H., and Godelle, B. (1997b) Role of mutator alleles in adaptive evolution. *Nature* **387**: 700–702.
Tainer, J.A., Thayer, M.M., and Cunningham, R.P. (1995) DNA repair proteins. *Curr Opin Struct Biol* **5**: 20–26.
Tajiri, T., Maki, H., and Sekiguchi, M. (1995) Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*. *Mutat Res* **336**: 257–267.
Tang, M., Shen, X., Frank, E.G., O'Donnell, M., Woodgate, R., and Goodman, M.F. (1999) UmuD'(2)C is an error-prone DNA polymerase, *Escherichia coli* pol V. *Proc Natl Acad Sci USA*. **96**: 8919–8924.

Tark, M., Tover, A., Tarassova, K., Tegova, R., Kivi, G., Hõrak, R., and Kivisaar, M. (2005) A DNA polymerase V homologue encoded by TOL plasmid pWW0 confers evolutionary fitness on *Pseudomonas putida* under conditions of environmental stress. *J Bacteriol* **187**: 5203–5213.

Tassotto, M.L., and Mathews, C.K. (2002) Assessing the metabolic function of the MutT 8-oxodeoxyguanosine triphosphatase in Escherichia coli by nucleotide pool analysis. *J Biol Chem.* **277**: 15807–15812.

Tchou, J., Kasai, H., Shibutani, S., Chung, M.H., Laval, J., Grollman, A.P., and Nishimura, S. (1991) 8-oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. *Proc Natl Acad Sci USA* **88**: 4690–4694.

Tchou, J., and Grollman, A.P. (1993) Repair of DNA containing the oxidatively-damaged base, 8-oxoguanine. *Mutat Res* **299**: 277–287.

Tegova, R., Tover, A., Tarassova, K., Tark, M., and Kivisaar, M. (2004) Involvement of Error-Prone DNA Polymerase IV in Stationary-Phase Mutagenesis in *Pseudomonas putida*. *J Bacteriol* **186**: 2735–2744.

Tenaillon, O., Denamur, E., and Matic, I. (2004) Evolutionary significance of stress-induced mutagenesis in bacteria. *Trends Microbiol* **12**: 264–270.

Thayer, M.M., Ahern, H., Xing, D., Cunningham, R.P., and Tainer, J.A. (1995) Novel DNA binding motifs in the DNA repair enzyme endonuclease III crystal structure. *EMBO J.* **14**: 4108–4120.

Theil, E.C., Matzapetakis, M., and Liu, X. (2006) Ferritins: iron/oxygen biominerals in protein nanocages. *J Biol Inorg Chem* **11**: 803–810.

Timms, A.R., Muriel, W., and Bridges, B.A. (1999) A UmuD,C-dependent pathway for spontaneous G:C to C:G transversions in stationary phase *Escherichia coli mut Y*. *Mutat Res* **435**: 77–80.

Tiraby, J.G., and Fox, M.S. (1973) Marker discrimination in transformation and mutation of pneumococcus. *Proc Natl Acad Sci USA* **70**: 3541–3545.

Tompkins, J.D., Nelson, J.L., Hazel, J.C., Leugers, S.L., Stumpf, J.D., and Foster, P.L. (2003) Error-prone polymerase, DNA polymerase IV, is responsible for transient hypermutation during adaptive mutation in *Escherichia coli*. *J Bacteriol* **185**: 3469–3472.

Torkelson, J., Harris, R.S., Lombardo, M.J., Nagendran, J., Thulin, C., and Rosenberg, S.M. (1997) Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation. *EMBO J* **16**: 3303–3311.

Tretyakova, N.Y., Niles, J.C., Burney, S., Wishnok, J.S., and Tannenbaum, S.R. (1999) Peroxynitrite-induced reactions of synthetic oligonucleotides containing 8-oxoguanine. *Chem Res Toxicol* **12**: 459–466.

Tretyakova, N.Y., Burney, S., Pamir, B., Wishnok, J.S., Dedon, P.C., Wogan, G.N., and Tannenbaum, S.R. (2000a) Peroxynitrite-induced DNA damage in the *supF* gene: correlation with the mutational spectrum. *Mutat Res* **447**: 287–303.

Tretyakova, N.Y., Wishnok, J.S., and Tannenbaum, S.R. (2000b) Peroxynitrite-induced secondary oxidative lesions at guanine nucleobases: chemical stability and recognition by the Fpg DNA repair enzyme. *Chem Res Toxicol* **13**: 658–664.

Trewick, S.C., Henshaw, T.F., Hausinger, R.P., Lindahl, T., and Sedgwick, B. (2002) Oxidative demethylation by *Escherichia coli* AlkB directly reverts DNA base damage. *Nature* **419**: 174–178.

Tsai-Wu, J.J., Liu, H.F., and Lu, A.L. (1992) *Escherichia coli* MutY protein has both N-glycosylase and apurinic/apyrimidinic endonuclease activities on A.C and A.G mispairs. *Proc Natl Acad Sci USA* **89**: 8779–8783.

Tsui, H.C., Feng, G., and Winkler, M.E. (1997) Negative regulation of *mutS* and *mutH* repair gene expression by the Hfq and RpoS global regulators of *Escherichia coli* K-12. *J Bacteriol.* **179**: 7476–7487.

Tudek, B. (2003) Imidazole ring-opened DNA purines and their biological significance. *J Biochem Mol Biol* **36**: 12–19.

Wagner, J., Gruz, P., Kim, S.R., Yamada, M., Matsui, K., Fuchs, R.P., and Nohmi, T. (1999) The *dinB* gene encodes a novel *E. coli* DNA polymerase, DNA pol IV, involved in mutagenesis. *Mol Cell* **4**: 281–286.

Wagner, J., and Nohmi, T. (2000) *Escherichia coli* DNA polymerase IV mutator activity: genetic requirements and mutational specificity. *J Bacteriol* **182**: 4587–4595.

Wallace, S.S. (1998) Enzymatic processing of radiation-induced free radical damage in DNA. *Radiat Res* **150**: S60–79.

Van Houten, B., Croteau, D.L., DellaVecchia, M.J., Wang, H., and Kisker, C. (2005) 'Close-fitting sleeves': DNA damage recognition by the UvrABC nuclease system. *Mutat Res* **577**: 92–117.

Wandersman, C., and Delepelaire, P. (2004) Bacterial iron sources: from siderophores to hemophores. *Annu Rev Microbiol* **58**: 611–647.

Wang, G., Alamuri, P., Humayun, M.Z., Taylor, D.E., and Maier, R.J. (2005) The *Helicobacter pylori* MutS protein confers protection from oxidative DNA damage. *Mol Microbiol* **58**: 166–176.

Wang, H., Yang, Y., Schofield, M.J., Du, C., Fridman, Y., Lee, S.D., Larson, E.D., Drummond, J.T., Alani, E., Hsieh, P., and Erie, D.A. (2003) DNA bending and unbending by MutS govern mismatch recognition and specificity. *Proc. Natl. Acad. Sci. USA*. **100**: 14822–14827. .

Wang, H., and Hays, J.B. (2004) Signaling from DNA mispairs to mismatch-repair excision sites despite intervening blockades. *EMBO J* **23**: 2126–2133.

Varshney, U., Hutcheon, T., and van de Sande, J.H. (1988) Sequence analysis, expression, and conservation of *Escherichia coli* uracil DNA glycosylase and its gene (*ung*). *J Biol Chem* **263**: 7776–7784.

Watson, M.E., Jr., Burns, J.L., and Smith, A.L. (2004) Hypermutable *Haemophilus influenzae* with mutations in *mutS* are found in cystic fibrosis sputum. *Microbiology* **150**: 2947–2958.

Weichart, D., Querfurth, N., Dreger, M., and Hengge-Aronis, R. (2003) Global role for ClpP-containing proteases in stationary-phase adaptation of *Escherichia coli*. *J Bacteriol* **185**: 115–125.

Weller, G.R., Kysela, B., Roy, R., Tonkin, L.M., Scanlan, E., Della, M., Devine, S.K., Day, J.P., Wilkinson, A., d'Adda di Fagagna, F., Devine, K.M., Bowater, R.P., Jeggo, P.A., Jackson, S.P., and Doherty, A.J. (2002) Identification of a DNA nonhomologous end-joining complex in bacteria. *Science* **297**: 1686–1689.

Venturi, V. (2003) Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different? *Mol Microbiol* **49**: 1–9.

Wiedenheft, B., Mosolf, J., Willits, D., Yeager, M., Dryden, K.A., Young, M., and Douglas, T. (2005) An archaeal antioxidant: characterization of a Dps-like protein from *Sulfolobus solfataricus*. *Proc Natl Acad Sci USA* **102**: 10551–10556.

Wilson, D.M., 3rd, and Bohr, V.A. (2007) The mechanics of base excision repair, and its relationship to aging and disease. *DNA Repair (Amst)* **6**: 544–559.
Wink, D.A., and Laval, J. (1994) The Fpg protein, a DNA repair enzyme, is inhibited by the biomediator nitric oxide in vitro and in vivo. *Carcinogenesis* **15**: 2125–2129.
Viswanathan, M., and Lovett, S.T. (1999) Exonuclease X of *Escherichia coli*. A novel 3'-5' DNase and Dnaq superfamily member involved in DNA repair. *J Biol Chem* **274**: 30094–30100.
Wolf, S.G., Frenkiel, D., Arad, T., Finkel, S.E., Kolter, R., and Minsky, A. (1999) DNA protection by stress-induced biocrystallization. *Nature* **400**: 83–85.
Volk, D.E., House, P.G., Thiviyanathan, V., Luxon, B.A., Zhang, S., Lloyd, R.S., and Gorenstein, D.G. (2000) Structural similarities between MutT and the C-terminal domain of MutY. *Biochemistry* **39**: 7331–7336.
Wood, M.L., Dizdaroglu, M., Gajewski, E., and Essigmann, J.M. (1990) Mechanistic studies of ionizing radiation and oxidative mutagenesis: genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in a viral genome. *Biochemistry* **29**: 7024–7032.
Wyrzykowski, J., and Volkert, M.R. (2003) The *Escherichia coli* methyl-directed mismatch repair system repairs base pairs containing oxidative lesions. *J Bacteriol* **185**: 1701–1704.
Xia, Z., Azurmendi, H.F., and Mildvan, A.S. (2005) Transient state kinetic studies of the MutT-catalyzed nucleoside triphosphate pyrophosphohydrolase reaction. *Biochemistry* **44**: 15334–15344.
Xu, W., Dunn, C.A., Jones, C.R., D'Souza, G., and Bessman, M.J. (2004) The 26 Nudix hydrolases of Bacillus cereus, a close relative of Bacillus anthracis. *J Biol Chem* **279**: 24861–24865.
Xu, W., Dunn, C.A., O'Handley S, F., Smith, D.L., and Bessman, M.J. (2006) Three new Nudix hydrolases from *Escherichia coli*. *J Biol Chem* **281**: 22794–22798.
Yamada, M., Nunoshiba, T., Shimizu, M., Gruz, P., Kamiya, H., Harashima, H., and Nohmi, T. (2006) Involvement of Y-family DNA polymerases in mutagenesis caused by oxidized nucleotides in *Escherichia coli*. *J Bacteriol.* **188**: 4992–4995.
Yamagata, Y., Kato, M., Odawara, K., Tokuno, Y., Nakashima, Y., Matsushima, N., Yasumura, K., Tomita, K., Ihara, K., Fujii, Y., Nakabeppu, Y., Sekiguchi, M., and Fujii, S. (1996) Three-dimensional structure of a DNA repair enzyme, 3-methyladenine DNA glycosylase II, from *Escherichia coli*. *Cell* **86**: 311–319.
Yamaguchi, M., Dao, V., and Modrich, P. (1998) MutS and MutL activate DNA helicase II in a mismatch-dependent manner. *J Biol Chem* **273**: 9197–9201.
Yamamoto, Y., Poole, L.B., Hantgan, R.R., and Kamio, Y. (2002) An iron-binding protein, Dpr, from *Streptococcus mutans* prevents iron-dependent hydroxyl radical formation in vitro. *J Bacteriol* **184**: 2931–2939.
Yang, W. (2003) Damage repair DNA polymerases Y. *Curr Opin Struct Biol* **13**: 23–30.
Yang, W. (2007) Human MutLalpha: the jack of all trades in MMR is also an endonuclease. *DNA Repair (Amst)* **6**: 135–139.
Yanofsky, C., Cox, E.C., and Horn, V. (1966) The unusual mutagenic specificity of an *E. coli* mutator gene. *Proc Natl Acad Sci USA* **55**: 274–281.
Yeiser, B., Pepper, E.D., Goodman, M.F., and Finkel, S.E. (2002) SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness. *Proc Natl Acad Sci USA* **99**: 8737–8741.

SUMMARY IN ESTONIAN

DNA paardumisvigade reparatsiooni ja DNA oksüdatiivsete kahjustuste kaitsesüsteemide roll statsionaarse faasi mutatsioonide ärahoidmisel bakteris *Pseudomonas putida*

Looduses elavad bakterid pidevalt muutuvates keskkonnatingimustes, kus nende kasv on pärsitud peamiselt toitainete vähesuse tõttu. Seega viibivad bakterid enamasti stressitingimustes, kus toimub pidev konkurents olemasolevate toitainevarude pärast. Taolises olukorras toimub bakteripopulatsiooni geneetiline kohastumine muutunud keskkonnatingimustega tänu bakterites tekkinud mutatsioonidele, mida nimetatakse kas statsionaarse faasi mutatsioonideks või stressist indutseeritud mutatsioonideks. Mõiste statsionaarse faasi mutagenees hõlmab endas erinevaid bakteris asetleidvaid mutageenseid mehhanisme, mis indutseeritakse stressivastusena mitmetele keskkonnatingimustele, mille tulemusena suureneb mutatsioonisagedus ning bakteripopulatsiooni geneetiline mitmekesisus, võimaldades antud keskkonnatingimustega paremini kohastunud mutantide teket.

Kuna looduslikes elupaikades elavad bakterid peamiselt toitainete puuduses, on enamus statsionaarse faasi mutatsiooniprotsesse kajastavatest uuringutest püüdnud matkida looduses esinevaid olukordi, kasutades selleks süsinikuallika või vajaliku aminohappe nälgimist. On näidatud, et stressitingimustes olevates bakterites aset leidvad mutageensed mehhanismid erinevad aktiivselt kasvavates bakterites toimuvatest. Samas, ühest rada statsionaarse faasi mutatsioonide tekkeks ilmselt pole. Nimelt võib mutatsioonisageduse tõus statsionaarses faasis olevates bakterites olla tingitud erinevatest mehhanismidest, mis peamiselt osalevad raku makromolekulide kaitsmisel ja/või reparatsioonis. Näiteks tõuseb vigu tegevate DNA polümeraaside poolt läbiviidava DNA sünteesi osakaal vastusena DNA kahjustus(t)ele nälgivates bakterites, mis viib omakorda olukorrani, kus suure hulga vigade tõttu ei suuda DNA reparatsioon enam efektiivselt toimida. Samuti suurendab geneetilist mitmekesisust stressitingimustes indutseeritud transposoonide ja insertsiooniliste elementide ümberpaiknemine genoomis (Ilves *et al.*, 2001; Shapiro, 2005). Lisaks eelpooltoodule on ka DNA oksüdatiivsed kahjustused üheks oluliseks statsionaarse faasi mutatsioonide tekke allikaks. Eriti suurt tähelepanu on pälvinud oksüdeeritud guaniin – 7,8-dihüdro-8-oksüguaniin (8-oksüG) tänu võimele paarduda adeniiniga, mis soodustab G:C→T:A transversioonide teket (Shibutani *et al.*, 1991). Hiljuti avastatud 8-oksüG edasise oksüdeerumise produktid on aga leitud olevat isegi mutageensemad kui 8-oksüG ise, kuna nad on suutelised DNA replikatsiooni peatama (David *et al.*, 2007). 8-oksüG mutageense potentsiaali alandamiseks on olemas oksüdeeritud guaniini (GO) reparatsiooni süsteem, milles osalevad DNA-st lämmastikaluseid väljalõikavad glükosülaasid. Juhul, kui 8-oksüG jääb

DNA-st või nukleotiidide seast eemaldamata, mutatsioonisagedus suureneb, ja seda ka nälgivates bakterites (Bridges *et al.*, 1996). Kõrgenenud mutatsioonisagedus ilmnes ka sel juhul kui *E. coli* rakkudes puudus statsionaarse faasispetsiifiline DNA-ga seostuv valk Dps, mis kaitseb genoomi muuhulgas ka oksüdatiivsete kahjustuste eest (Martinez and Kolter, 1997). Antud katses oli aga rakkudele lisatud ka vesinikperoksiidi. Seega ei ole selge, kas Dps mõjutab statsionaarse faasi mutatsioonide tekkesagedust või mitte.

Vastusena DNA kahjustus(t)ele indutseeritakse ka spetsialiseeritud DNA polümeraasid Pol IV, Pol V ja Pol II, millest Pol IV ja Pol V on vigutegevad (*error-prone*) (Friedberg *et al.*, 2002). DNA Pol IV ja Pol V poolt läbiviidav mutageenne DNA süntees suurendab vastavalt raaminihke-mutatsioonide ja asendusmutatsioonide tekkesagedust nälgivates bakterites (Bhamre *et al.*, 2001; McKenzie *et al.*, 2001).

Sellal kui vigaderohke DNA sünteesi tagajärjel geneetiline mitmekesisus suureneb, on DNA reparatsioonil mutatsioonide teket ärahoidev ehk hoopis vastupidine roll. Seega sõltub mutatsioonisagedus ka DNA reparatsiooni efektiivsusest. Lisaks ülalmainitud GO reparatsioonile on näidatud ka ühe põhilisema, peamiselt DNA replikatsioonivigu korrigeeriva paardumisvigade reparatsiooni (*mismatch repair*, MMR) osalust statsionaarse faasi mutageneesis. Aktiivset diskussiooni on põhjustanud teema, mille kohaselt MMR alatalitlus võiks soodustada statsionaarse faasi mutatsioonide teket nälgivates bakterites (Foster, 1999b; Harris *et al.*, 1999). Samuti on arvatud, et replikatsioonivigade esinemissageduse suurenemine nälgivates bakterites viib olukorrani, kus MMR valkude hulk jääb liiga madalaks, et kõiki vigu korrigeerida (Negishi *et al.*, 2002).

Stressi poolt indutseeritud mutageneesi mehhanisme on siiani uuritud peamiselt *E. coli* mudelsüsteemide baasil. Paraku pole kõik bakteris *E. coli* avastatud mutageneesimehhanismid alati üks-üheselt ülekantavad teistele bakteritele. Meie uurimisrühm kasutab mutatsiooniprotsesside väljaselgitamiseks nälgivates mikroobides mudelina bakterit *Pseudomonas putida*. Pseudomonaadid on looduses laialt levinud, ning võimelised kiiresti evolutsioneeruma erinevates elukeskkondades nagu veekogud, muld, aga ka looma- ja taimekoed. Seega võib mutatsiooniprotsesside mehhanismide uurimine *P. putida* näitel anda laiemat teavet evolutsioneerumisstrateegiate kohta stressitingimuses viibivates bakterites. Meie laboris teostatud varasemad uuringud viitasid mutatsiooniprotsesside erinevusele kasvavates ja nälgivates *P. putida* rakkudes (Kasak *et al.*, 1997). Sellest tulenevalt sai antud töö eesmärgiks uurida stressi poolt indutseeritud mutatsiooniprotsesside molekulaarseid mehhanisme süsinikuallika näljas oleva mullabakteri *P. putida* näitel. Põhiliseks eesmärgiks oli uurida oksüdatiivsete DNA kahjustuste mõju ning oksüdatiivsete kahjustuste eest kaitsvate süsteemide, peaasjalikult oksüdeeritud guaniini reparatsiooni rolli statsionaarse faasi mutatsioonide tekkele bakteris *P. putida*. Lisaks seati

eesmärgiks selgitada välja paardumisvigade reparatsiooni osalus mutatsioonide ärahoidmisel nälgivates *P. putida* rakkudes.

Töös esitatud tulemused võib kokku võtta järgnevalt:

1. Mutatsioonitekke mehhanismid on eksponentsiaalselt kasvavates ja süsinikuallika näljas olevates *P. putida* rakkudes erinevad. Erinevused ilmnesid ka vaadeldud nälgimisperioodi kestel, mida kinnitasid muutused mutatsioonispektris. Nälgimise alg-perioodil oli kõige sagedam mutatsioonitüüp C→A transversioon, samas C→A asendusmutatsiooni osakaal nälgimise jooksul langes ning väikeste 2–3 nt deletsioonide ja insertsioonide osakaal suurenes. Väikeste deletsioonide ja insertsioonide teke oli positiivselt reguleeritud statsionaarse faasi sigma faktori RpoS poolt.
2. DNA oksüdatiivsed kahjustused on oluliseks statsionaarse faasi mutatsioonide tekkepõhjuseks nälgivates *P. putida* rakkudes. Oksüdeeritud guaniini reparatsiooniraja DNA glükosülaasid on mutatsioonide ärahoidmisel funktsionaalsed kogu vaadeldud nälgimisperioodi kestel. MutY või MutM defektsus nälgivates *P. putida* rakkudes suurendas G→T transversioonide esinemissagedust, mis näitab, et nende valkude funktsioon *P. putida* rakkudes sarnaneb mudelorganismi *E. coli* vastavate valkude funktsiooniga. DNA glükosülaasi MutY kodeeriva geeni inaktiveerimine suurendas asendusmutatsioonide tekkesagedust olenemata *P. putida* kasvufaasist. DNA glükosülaasi MutM puudumisel ei suurenenud mutatsioonisagedus võrreldes algtüvega oluliselt ei kasvavates ega ka nälgivates rakkudes, kuigi G→T transversioonide osakaalu tõus nälgivates rakkudes viitab sellele, et MutM valk on nälgivates rakkudes siiski funktsionaalne. Võimalik, et MutM valgu puudumisel asendab teda mõni teine DNA glükosülaas, näiteks Nth valgu homoloog.
3. Pürofosfohüdrolaasi MutT mõju mutatsioonide ärahoidmisele sõltus *P. putida* kasvufaasist. Nälgivates *P. putida* rakkudes ilmnes MutT defektsuse korral väga tugev mutaatorfenotüüp ja mutatsioonispektri analüüs näitas, et suurenenud oli A→C mutatsioonide esinemissagedus, mis näitab, et sarnaselt mudelorganismiga *E. coli* elimineerib ka *P. putida* MutT oksüdeeritud dGTP-d nukleotiidide kogumist. Funktsionaalse MutT valgu puudumine *P. putida* kasvavates rakkudes mutatsioonisagedust oluliselt ei mõjutanud. Oletame, et kasvavates rakkudes supresseerib mõni Nudix hüdrolaasidest MutT-defektsete bakterite mutaatorfenotüübi.
4. Lisaks oksüdeeritud guaniinile võib ka oksüdatiivselt kahjustatud adeniini olemasolu DNA-s mõjutada statsionaarse faasi mutatsioonide teket nälgivates *P. putida* rakkudes. Vastupidiselt ootustele me guaniini edasise oksüdeerumise produktide osalust mutageneesis ei tuvastanud.
5. Meie töö tulemused näitasid, et Dps (*DNA binding protein from starved cells*) valk, mis kaitseb genoomi oksüdatiivsete kahjustuste eest ei osale statsionaarse faasi mutatsioonide ärahoidmisel *P. putida* nälgivates rakkudes.

Funktsionaalne Dps valk kaitseb siiski *P. putida* genoomi rakuväliste DNA-d oksüdatiivselt kahjustavate ühendite eest.

6. Kuigi kirjandusest võib leida andmeid selle kohta, et üheks võimalikuks statsionaarse kasvufaasi mutageneesi mehhanismiks võiks olla paardumis-vigade reparatsiooni efektiivsuse langus bakterite pikemaajalises näljastressis viibimisel, meie katsetulemused seda ei kinnita. MMR puudusel täheldasime ligikaudu 25-kordset asendusmutatsioonide tekkesageduse suurenemist nälgivates rakkudes vaadeldud 2-nädalase nälgimisperioodi vältel. Mutatsioonide analüüsil nägime, et algtüve ja MMR-defektsete mutantide mutatsioonispekter oli erinev, mis samuti kinnitas, et MMR on statsionaarse faasi rakkudes funktsionaalne. Seega, kuna mutatsioonide tekkimist nälgimise ajal MMR alatalitlusega seostada ei saanud, peab statsionaarse faasi mutatsioonide tekkimiseks olema teisi võimalusi.

Eelpooltoodu põhjal võib järeldada, et nälgiv *P. putida* populatsioon on üsna dünaamiline ning statsionaarse faasi mutatsioonide tekkemehhanismid muutuvad ka sõltuvalt sellest, kui kaua on bakterid nälginud. Nii oksüdatiivsed DNA kahjustused, kui ka ebatäpne DNA replikatsioon soodustavad statsionaarse faasi mutatsioonide teket. Samas GO reparatsiooni ja MMR funktsionaalsus nälgimise vältel ei vähenenud, mis välistab DNA reparatsiooni alatalitluse kui ühe võimaliku statsionaarse faasi mutatsiooni tekkemehhanismi bakteris *P. putida*. Kuna kõik protsessid, mis statsionaarse faasi mutatsioonide tekkimist mõjutavad, on omavahel seotud, käsitles antud töö ainult ühte osa keerukast mutageneesi masinavärgist. Seega pakub statsionaarse faasi mutatsiooni mehhanismide väljaselgitamine jätkuvalt võimalusi uuteks avastusteks ka tulevikuks.

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PUBLICATIONS

I

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Different Spectra of Stationary-Phase Mutations in Early-Arising versus Late-Arising Mutants of *Pseudomonas putida*: Involvement of the DNA Repair Enzyme MutY and the Stationary-Phase Sigma Factor RpoS

Signe Saumaa, Andres Tover, Lagle Kasak,† and Maia Kivisaar*

Department of Genetics, Institute of Molecular and Cell Biology, Tartu University and Estonian Biocentre, 51010 Tartu, Estonia

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Stationary-phase mutations occur in populations of stressed, nongrowing, and slowly growing cells and allow mutant bacteria to overcome growth barriers. Mutational processes in starving cells are different from those occurring in growing bacteria. Here, we present evidence that changes in mutational processes also take place during starvation of bacteria. Our test system for selection of mutants based on creation of functional promoters for the transcriptional activation of the phenol degradation genes *pheBA* in starving *Pseudomonas putida* enables us to study base substitutions (C-to-A or G-to-T transversions), deletions, and insertions. We observed changes in the spectrum of promoter-creating mutations during prolonged starvation of *Pseudomonas putida* on phenol minimal plates. One particular C-to-A transversion was the prevailing mutation in starving cells. However, with increasing time of starvation, the importance of this mutation decreased but the percentage of other types of mutations, such as 2- to 3-bp deletions, increased. The rate of transversions was markedly elevated in the *P. putida* MutY-defective strain. The occurrence of 2- to 3-bp deletions required the stationary-phase sigma factor RpoS, which indicates that some mutagenic pathway is positively controlled by RpoS in *P. putida*.

Natural microbial populations spend most of their life under nutrient deprivation due to intense competition for available resources. Under these limiting conditions, there is selective pressure for any mutation that confers a competitive advantage. Differences in the spectra of mutations have been observed between sets of mutants appearing in starving bacterial populations under selective conditions and in actively growing bacterial cultures (22, 32, 52, 55). The starvation conditions encountered during stationary-phase incubation may permit a transient increase in the mutation rate due to a variety of factors, including decreased fidelity during replication and reduction of DNA repair activity (9, 18, 58, 65; see also references in reference 21). Another mechanism that may increase genetic diversity is the movement of transposable elements (14, 56). Also, some data (51) indicate that different mutagenic pathways might be involved in mutation processes creating either early- or late-arising mutants in the stationary-phase cell population.

It has been suggested that the methyl-directed mismatch repair system might be limiting in stationary phase and nutritionally deprived cells, giving rise to stationary-phase mutations (19, 22, 54, 55). However, the role of mismatch repair in stationary-phase mutations is controversial (20, 26). Bridges et al. (12) proposed that in nongrowing bacteria, oxidized guanine residues, including 7,8-dihydro-8-oxoguanine, constitute an important component of spontaneous mutation. Pairing of adenine with 7,8-dihydro-8-oxoguanine, an oxidatively damaged form of guanine, is known to cause G:C to T:A transversions during DNA synthesis (41). The DNA repair enzymes MutY and MutM are part of a multiple line of defenses against oxidative damage to DNA (42). Cells that lack active MutY protein have elevated rates of G:C to T:A transversions (45). In *Escherichia coli*, accumulation of prototrophic mutants during amino acid starvation was caused by 7,8-dihydro-8-oxoguanine, and the rate of reversions enabling a prototrophic phenotype in starved cells was remarkably elevated in MutY-defective strains (12).

Knowledge about mechanisms of mutational processes in starving bacteria is mostly based on investigations of *E. coli* (21). The genus *Pseudomonas* is a diverse and ecologically significant group of bacteria (59), but there are only a few examples of studies on mutagenesis in *Pseudomonas* spp. (32, 35, 38, 48, 49; see also references in reference 21). Our test system employing a promoterless *pheBA* cluster in plasmid pEST1414 as a reporter enables us to isolate and characterize mutations that create functional promoters for the transcription of the *pheBA* genes in *Pseudomonas putida* (32). The *pheBA* genes encode catechol 1,2-dioxygenase and phenol monooxygenase, respectively (33). When the *pheBA*-expressing plasmid is introduced into phenol-nondegrading *P. putida* strain PaW85, bacteria gain the ability to utilize phenol as a sole carbon source. We have shown that promoters for the transcription of the initially promoterless phenol degradation genes *pheBA* were created as a result of base substitutions,

* Corresponding author. Mailing address: Department of Genetics, Institute of Molecular and Cell Biology, Tartu University and Estonian Biocentre, 23 Riia Street, 51010 Tartu, Estonia. Phone: 372 7 375015. Fax: 372 7 420286. E-mail: maiak@ebc.ee.

† Present address: Department of Molecular Genetics, National Institute of Chemical Physics and Biophysics, 12618 Tallinn, Estonia.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or construction	Source or reference
E. coli		
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	15
CC118 λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λpir phage lysogen	29
P. putida		
PaW85	Tn*4652*	5
PKS54	Tn*4652 rpoS*::*km*	47
PaW85 *mutY*::*tet*	Tn*4652 mutY*::*tet*	This work
Plasmids		
pEST1414	Plasmid pAYC32 carrying promoterless *pheBA* operon	32
pBluescript SK(+)	Cloning vector (Ap^r)	Stratagene
pBR322	Cloning vector (Ap^r Tet^r)	8
pBlscrMutY	pBluescript SK(+) containing PCR-amplified *P. putida mutY* gene cloned into *Eco*RV- and *Sma*I-cleaved vector	This work
pBLscrMutY-Tet^r	*tet* gene from pBR322 inserted into *Eco*RI-*Eco*47III-cleaved *mutY* gene in pBLscrMutY	This work
pGP704 L	Delivery plasmid for homologous recombination	50
pGPMutY-Tet^r	*mutY*::*tet*-sequence-containing *Sac*I-*Kpn*I fragment from pBlscrMutY-Tet cloned into pGP704L	This work
pRK2013	Helper plasmid for conjugal transfer of pGP704L	16

deletions, or transposition of the mobile DNA elements Tn*4652* and IS*1411* (31, 32).

Previous results (32) indicated that different mechanisms are responsible for the appearance of mutations in exponentially growing and stationary-phase cells of *P. putida*. The accumulation rate of the Phe$^+$ mutants on selective plates was found to be dependent on the physiological state of the bacteria before plating: the accumulation was much higher for bacteria plated from stationary-phase culture than for those plated from exponentially growing cells. Also, we found that stationary-phase mutants appeared mainly due to one particular C-to-A base substitution, whereas different deletions (mostly in the range of 6 to 40 bp) prevailed in cultures growing exponentially.

In this report, we focused on studies of the effect of time of starvation on the spectrum of stationary-phase mutations. It appeared that the spectrum of stationary-phase mutations among early-arising mutants differed from that of the later-arising ones. We observed that one particular C-to-A transversion was the dominant mutation type in the cell population at the beginning of starvation, but the proportion of the other type of mutations, 2- to 3-bp deletions, increased remarkably with time of starvation. The formation of 2- to 3-bp deletions required stationary-phase sigma factor RpoS, whereas the frequency of occurrence of transversions was affected by the functionality of the 7,8-dihydro-8-oxoguanine repair enzyme MutY. The balance between various processes involved in mutagenesis in starving cells will be discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. Complete medium was Luria-Bertani (LB) medium (44), and minimal medium was M9 (1). Phenol minimal plates with 1.5% Difco agar contained 2.5 mM phenol as the sole carbon and energy source. Antibiotics were added at the following final concentrations: for *E. coli*, ampicillin at 100 μg/ml; for *P. putida*, carbenicillin at 1,000 to 3,000 μg/ml; for both organisms, kanamycin at 50 μg/ml, tetracycline at 10 μg/ml, and rifampin at 100 μg/ml. *E. coli* was incubated at 37°C, and *P. putida* was incubated at 30°C. *E. coli* was transformed with plasmid DNA as described by Hanahan (25). *P. putida* was electrotransformed as described by Sharma and Schimke (57). *E. coli* TG1 (15) was used for the DNA cloning procedures.

Isolation of Phe$^+$ mutants. Independent cultures of *P. putida* strains carrying plasmid pEST1414 were generated by growing cells to saturation in LB medium, diluting this culture by 10^6 into fresh LB medium, dispensing 2-ml aliquots into test tubes, and allowing cells again to reach saturation by growing cells for 16 to 18 h. Then 0.5-ml samples (approximately 5×10^8 cells) were harvested by centrifugation, washed with M9 solution, and plated on phenol minimal plates. When different amounts of pEST1414-carrying cells of the same cultures were plated on selective plates, they were plated with equal amounts of scavenger cells (approximately 10^9 cells). Scavenger cells (*P. putida* PaW85 carrying a pEST1414 derivative lacking the *pheA* coding sequence) were grown to saturation in LB medium and concentrated by centrifugation and resuspension in 0.1 volume of M9 solution.

To analyze the Phe$^+$ mutants from the growing cultures, independent Phe$^+$ colonies appearing on selective plates on day 2 were picked from separate plates and used for DNA sequence analysis of the plasmids conferring constitutive expression of the *pheBA* genes. To characterize mutations occurring in starving cultures, the Phe$^+$ mutants accumulating on selective plates on day 3 and later were analyzed. To exclude the copies of original, wild-type (Phe$^-$) plasmids present in the Phe$^+$ colonies, we isolated plasmid DNA from the mutants and transformed *E. coli* TG1, selecting for resistance to ampicillin. Transformants were assayed for expression of the *pheB* gene by testing expression of catechol 1,2-dioxygenase as described before (27) except that the measurements were carried out in cell suspensions.

Analysis of insertions of Tn*4652* and IS*1411* into pEST1414. Constitutively expressed fusion promoters are created as a result of transposition of transposon Tn*4652* upstream of the *pheA* coding sequence (46). Kasak et al. have previously shown that transposon insertions account for one-third of all stationary-phase mutations in bacteria carrying plasmid pEST1414 (32). Two primers, pheA (5′-TGCTCAAGATTATCATTACGCT-3′), complementary to the *pheA* coding sequence at nucleotides 11 to 32, and TnR (5′-ATCAGCATAGACGGCTAG CCAG-3′), complementary to the right end of Tn*4652* at nucleotides 101 to 122, were used to amplify the Tn*4652* insertion regions in Phe$^+$ cells containing hybrid plasmids. Nurk et al. have previously shown (46) that fusion promoters are preferentially created by the right-end sequence of the transposon. Therefore, the detection scheme used here was designed to reveal promoters generated by fusion of the right end of the element with the upstream sequences of the *pheA* gene. IS*1411* can activate the promoterless *pheBA* genes due to outward-directed promoters on its left end (31). The oligonucleotide ORF2 (5′-CGAG GTTATTCAGTT-3′), complementary to nucleotides 47 to 61 relative to the start codon of the *tnpA* gene of IS*1411*, and oligonucleotide pheA were used for PCR analysis of Phe$^+$ mutants for insertions of IS*1411* upstream of the *pheA* gene.

DNA sequence analysis. The ~250-bp DNA region of the Phe$^+$ mutants upstream of the *pheBA* genes in plasmid pEST1414 was analyzed by DNA sequencing. The DNA segment containing this region was amplified by PCR with the oligonucleotides PAYC32 (5′-CTCGACCTTTGAGCCAAATG-3′) and CAT2,1 (5′-TTTTAACAGTCATAATTACTCTCTC-3′), complementary to the sequences of vector plasmid pAYC32 upstream of the *Sac*I site and to the

sequence −12 upstream of the *pheB* initiator codon, respectively. The nucleotide sequences were determined with the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech Inc.). The oligonucleotide used in the sequencing of the mutant DNA region upstream of the *pheBA* genes in plasmid pEST1414 was either CAT2,1 or AB0 (5′-GGAAGTATGCTTGGC-3′), complementary to the sequences −136 nucleotides upstream of the *pheB* initiator codon. The DNA sequencing reactions were analyzed with an ABI Prism 377 DNA sequencer (Perkin Elmer).

Construction of *P. putida* PaW85 *mutY* knockout mutant. The *mutY* gene sequence of *P. putida* was obtained by searching for *mutY* homologs in the unfinished *P. putida* KT2440 Genome Project website (http://www.tigr.org). The *mutY* gene was amplified by PCR from genomic DNA of *P. putida* PaW85 which is isogenic to *P. putida* strain KT2440. Two primers, KTYFw (5′-GCGCTCAA GGGGCTGTTCAAC-3′) and KTYRev (5′-CGGTGCGGGTCATCGGGCGT-3′), complementary to the sequences −23 nucleotides upstream of the ATG initiator codon and 9 nucleotides downstream of the TAG stop codon of the *P. putida mutY* gene, respectively, were used for DNA amplification in a standard PCR (96°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min; 25 cycles). The amplified DNA fragment containing the *mutY* gene was subcloned into pBluescript SK(+) cleaved with *Eco*RV and *Sma*I to obtain pBlscrMutY. The *Eco*RI-*Van*9I DNA fragment containing the *tet* gene from pBR322 was inserted into the *Eco*RI- and *Eco*47III-cleaved *mutY* gene. The resulting *mutY-tet* sequence from pBlscrMutY-Tet was inserted into plasmid pGP704L (50) by using the *Sac*I and *Kpn*I sites. pGPMutY-Tet was selected in *E. coli* strain CC118 λpir (29).

The interrupted *mutY* gene was inserted into the chromosome of *P. putida* PaW85 by homologous recombination. Plasmid pGPMutY-Tet, which does not replicate in hosts other than *E. coli* CC118 λpir, was conjugatively transferred into *P. putida* PaW85 by using helper plasmid RK2013 (16). The PaW85 *mutY::tet* knockout was verified by PCR analysis. In addition, the mutator phenotype of the clones containing the interrupted *mutY* sequence but lacking the original sequence was examined by measuring the spontaneous frequency of mutation to rifampin resistance.

Measurement of mutation frequency. The spontaneous mutation frequency of *P. putida* wild-type and MutY-defective strains was measured by calculating the average number of mutants (±standard deviation) per 10^9 cells. At least 20 independent cultures grown in LB as described above were plated on either rifampin or phenol minimal plates. Phe^+ colonies appearing on phenol minimal plates on day 2 were counted to estimate Phe^+ mutation frequency in growing cells.

Measurement of viability of MutY-defective *P. putida* on phenol minimal plates. The growth conditions for bacteria were the same as described for the isolation of Phe^+ mutants. About 5×10^8 M9-washed cells of *P. putida* wild-type strain PaW85 and its MutY-defective derivative were plated onto four phenol minimal plates, and small plugs were cut from the agar on each day of starvation. Bacteria from the plugs were suspended in M9 solution, and the number of CFU was determined on 0.1% glucose minimal plates.

RESULTS

Changes in spectrum of mutations during starvation. Previous experiments demonstrated that promoters for the transcriptional activation of the *pheBA* genes were created as a result of base substitutions, deletions, and transpositions of transposon Tn*4652* (32). In the article by Kasak et al. (32), we focused on differences between mutations occurring in growing cells and in stationary-phase cells but did not pay attention to whether the spectrum of stationary-phase mutations might be influenced by the duration of starvation. In the current report, mutant colonies that appeared on the phenol selective plates on days 3 to 7 after plating were analyzed on each separate day.

The accumulation curve of the Phe^+ mutants in starving cultures of *P. putida* wild-type strain PaW85 carrying the promoterless reporter plasmid pEST1414 is shown in Fig. 1. Approximately 60 to 70 Phe^+ mutants per day were subjected to sequence analysis. Mutants that appeared due to insertions of Tn*4652* (they accounted for one-third of all stationary-phase mutations) were excluded from this analysis (see Materials and Methods). The 250-bp sequence upstream of the *pheBA* genes

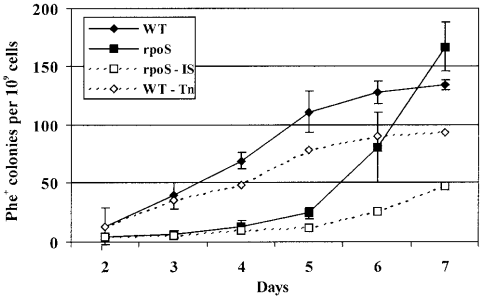

FIG. 1. Accumulation of Phe^+ mutants on phenol minimal plates in *P. putida* wild-type strain PaW85 (WT) and its RpoS-defective derivative PKS54 (rpoS). Dashed lines indicate the theoretical appearance of mutants (deduced from the results of PCR analysis of Tn*4652* and IS*1411* insertions in Phe^+ mutants) in the wild-type strain if Tn*4652*-linked mutants are subtracted (WT − Tn) and in PKS54 if IS*1411*-linked mutants are subtracted (rpoS − IS). Each point represents the mean of at least five independent determinations, and error bars represent standard deviations. Ilves et al. have previously shown that survival of the wild-type *P. putida* strain and its RpoS-defective mutant is not decreased during the first 5 days of starvation of bacteria on phenol minimal plates (30).

containing raw material for promoter creation (Fig. 2) was analyzed in mutant plasmids. Kasak et al. have previously shown that Phe^+ mutants that appeared on selective plates on day 2 contained mutations that occurred before plating in growing cultures, whereas colonies that emerged on phenol minimal plates on day 3 and later contained mutations that occurred after the cells were plated (32).

The results shown in Table 2 reveal changes in the spectrum of promoter-creating mutations during starvation. We observed a significant decline in the percentage of C-to-A transversion when the spectrum of the mutations of the Phe^+ colonies isolated on either day 3 or day 4 was compared to that of the mutants that appeared on day 7 ($\chi^2 = 22$ and 5.5, respectively; $P < 0.01$). Comparison of the spectra of early-arising and late-arising Phe^+ mutants revealed remarkable changes in the nature of the deletions occurring at different stages of starvation (Table 2). Deletions in the range of 6 to 45 bp were rarely represented among the mutants that appeared on days 3 to 5 and were not observed later. At the same time, the amount of smaller, 2- to 3-bp deletions increased among the promoter-creating mutations with time of the starvation. The proportion of 2- to 3-bp deletions was significantly different when the spectrum of the Phe^+ mutants that emerged on days 3 and 4 was compared to that of the mutants that appeared on day 7 ($\chi^2 = 11$, $P < 0.001$). On each day except day 5, we also detected another type of changes, formation of 2- to 3-bp insertions (Table 2). However, as shown in Table 2, in comparison with the deletions, the insertions occurred quite rarely. Every day, approximately 20 to 30% of the mutations remained undefined (Table 2) because they occurred outside the sequencing window.

Kasak et al. have previously shown that deletions in the range of 6 to 45 bp were characteristic of the Phe^+ mutations occurring in the growing cultures of *P. putida* PaW85 (32). A

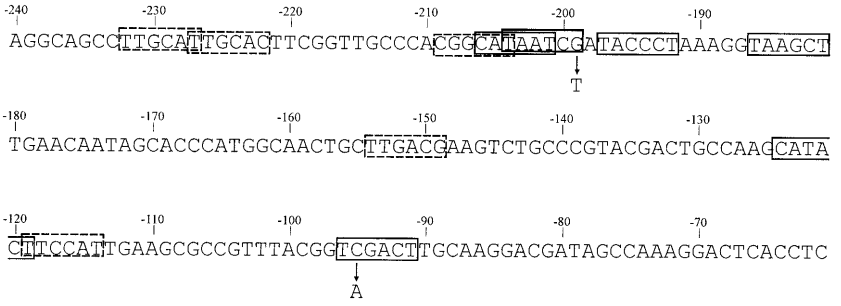

FIG. 2. Sequence upstream of *pheB* in plasmid pEST1414 contains raw material for promoter creation. The potential −35 and −10 hexamers of promoters are framed, −35 hexamers by dashed lines and −10 hexamers by continuous lines. The G-to-T and C-to-A transversions are marked by arrows. As shown in previous studies (32), base substitutions, deletions, and insertions can create promoter sequences similar to that of the *E. coli* RNA polymerase σ^{70} promoter consensus sequence TTGACAN$_{16\text{-}18}$TATAAT.

similar pattern of deletions was also revealed in the current study (Table 2). Only a few Phe$^+$ mutants appearing on day 2 were created by the 2- to 3-bp deletions, but the number of these deletions increased significantly among the mutations characterized on days 3 to 7 ($\chi^2 = 4$, $P = 0.04$). Thus, the occurrence of 2- to 3-bp deletions seems to be induced during long-term starvation.

Deficiency of MutY leads to increased frequency of C-to-A and G-to-T transversions in *P. putida*. There are several reports demonstrating that the lack of DNA repair enzyme MutY leads to an increased frequency of G:C to T:A transversions (41, 45). Moreover, in the case of the *E. coli mutY trpA23* strain, prototrophic mutants containing either G-to-T transversions at the *trpA23* site or small in-frame deletions in the *trpA* gene arise at an elevated rate when the bacteria are incubated under starvation conditions (11).

In order to study whether the *mutY* background would affect the nature and frequency of the appearance of Phe$^+$ mutants, we constructed a MutY-defective *P. putida* strain. The *mutY* gene sequence of *P. putida* was obtained from the unfinished *P. putida* KT2440 Genome Project website. The 1,065-nucleotide open reading frame of *P. putida mutY* encodes 355 amino acids. The deduced amino acid sequence of *P. putida* MutY revealed 78% identity with the putative MutY of *Pseudomonas aeruginosa* and 57% identity with *E. coli* and *Salmonella enterica* serovar Typhimurium MutY sequences (data not shown).

The *mutY* gene of *P. putida* was disrupted by the tetracycline resistance-encoding gene, and the *mutY*::*tet* sequence was used to replace the wild-type *mutY* by homologous recombination. The frequency of rifampin resistance mutations in wild-type and MutY-defective *P. putida* strains was examined by plating independent cultures on rifampin plates (Materials and Methods). The mutation frequency increased approximately 60-fold in the MutY-defective strain compared to the wild-type strain: the average number of Rif^r colonies per 10^9 cells was 1.9 ± 1.5 in the case of the wild-type strain and 120 ± 92 in the case of PaW85 *mutY*::*tet*. This demonstrates that lack of MutY leads to the mutator phenotype of *P. putida*.

We studied the frequency and spectrum of Phe$^+$ mutations in growing cells of *P. putida* PaW85 *mutY*::*tet*. Approximately 5×10^8 cells carrying the reporter plasmid pEST1414 were plated onto phenol minimal plates. Comparison of the wild-type and MutY-defective strains revealed about a 50-fold increase in the frequency of promoter-creating mutations in PaW85 *mutY*::*tet* when growing cultures were examined; the average number of Phe$^+$ colonies per 10^9 cells was 7.5 ± 5 in the case of the wild-type strain and 380 ± 220 in the case of the MutY-defective strain.

The Phe$^+$ mutants collected from separate selective plates in

TABLE 2. Effect of duration of starvation of *P. putida* cells on spectrum of Phe$^+$ mutations

Day of appearance of mutants	No. of mutants analyzed	No. (%) of mutants with:					
		C-to-A transversion	G-to-T transversion	Deletions		Insertions (2 to 3 nt)	Undefined mutations
				6 to 45 nt[b]	2 to 3 nt		
2[a]	77	40 (52)	14 (18)	16 (21)	2 (2.5)	3 (4)	2 (2.5)
3	69	47 (68)	4 (6)	4 (6)	0	2 (3)	12 (17)
4	62	30 (48)	10 (16)	2 (3)	5 (8)	3 (5)	12 (20)
5	70	30 (43)	9 (13)	3 (4)	6 (9)	0	22 (31)
6	60	18 (30)	12 (20)	0	13 (22)	1 (2)	16 (26)
7	61	16 (26)	11 (18)	0	12 (20)	5 (8)	17 (28)

[a] Phe$^+$ mutants appearing on selective plates on day 2 contained mutations that occurred in growing cultures before plating.
[b] nt, nucleotides.

TABLE 3. Spectrum of Phe^+ mutations occurring in the MutY-defective *P. putida* strain

Day	No. of mutants analyzed	No. (%) of mutants with:		
		C-to-A transversion	G-to-T transversion	Undefined mutation
2[a]	45	30 (67)	15 (33)	0
2[b]	72	57 (80)	15 (20)	0
3	44	27 (61)	15 (34)	2 (5)
6	40	12 (30)	10 (25)	18 (45)

[a] Experiment I; one mutant from each of 45 separate cultures was analyzed.
[b] Experiment II; four mutants from each of 18 separate cultures were analyzed.

two parallel experiments (experiments I and II) were subjected to further characterization. The spectra of mutants obtained from two studies (Table 3) were statistically similar ($\chi^2 = 1.7$, $P = 0.2$). The results shown in Table 3 demonstrated that all mutants characterized in the *mutY*::*tet* background contained either a C-to-A or G-to-T transversion at the same sites as in the mutants characterized by us before (Fig. 2). No other type of mutations (deletions, insertions) or Tn*4652* insertions could be detected. We observed a difference in the mutation frequency at the two different sites: the promoter-creating C-to-A mutation was represented at a higher frequency than the G-to-T mutation in both experiments.

The average number of Phe^+ mutants accumulating on phenol minimal plates per day per 5×10^8 plated *P. putida* MutY-defective cells decreased rapidly during starvation (Table 4). The lack of MutY causes the mutator phenotype of *P. putida*, and this could increase the frequency of lethal mutations in the cell population. Therefore, we studied survival of MutY-defective cells on phenol minimal plates (Materials and Methods). There were no differences in viability between the wild-type and *mutY*::*tet*^{r} strain during the first 6 days of starvation (data not shown). Instantly, survival of the MutY-defective strain decreased 100-fold for day 7 (5×10^6 cells per plate) and thereafter remained constant over the next week of starvation. Thus, the rapid decline in the rate of accumulation of Phe^+ mutants on selective plates was not caused by the death of bacteria. Rather, this could be explained by inhibition of accumulation of the mutants due to the high number of mutant colonies already present on selective plates.

To control whether the high number of the Phe^+ mutants per plate would inhibit subsequent accumulation of mutants in the MutY-deficient background, we repeated the experiment by plating different amounts (5×10^8, 5×10^7, and 5×10^6) of cells on selective plates. Cells of the wild-type *P. putida* strain and PaW85 *mutY*::*tet* were plated onto phenol minimal plates with approximately 10^9 scavenger cells (Materials and Methods). The data shown in Table 4 clearly demonstrate that the large number of Phe^+ colonies on selective plates inhibited further accumulation of the mutants. The accumulation of Phe^+ mutants was not inhibited if we plated 100 times fewer MutY-defective cells. The inhibition effect became apparent even in this case when 5×10^7 MutY-defective cells were plated (Table 4, MutY results, compare days 4 and 5 with day 6).

At the same time, the data presented in Table 4 show that the frequency of appearance of Phe^+ mutants was remarkably higher in the MutY-defective strain compared to that in the wild-type strain during all 7 days studied. The Phe^+ mutants that accumulated when fewer cells (5×10^6) of the MutY-defective strain were plated were subjected to further characterization. The spectrum of mutations that appeared on selective plates on days 3 and day 6 is shown in Table 3. Most of the Phe^+ mutants analyzed contained either C-to-A or G-to-T transversions. We did not detect deletions among the mutants studied. Some mutants, however, contained changes outside the sequencing window. The proportion of this type of mutant increased during the starvation period from 5% among colonies that appeared in day 3 on selective plates to 45% among those that arose on day 6.

Lack of stationary-phase sigma factor RpoS affects the spectrum of promoter-creating mutations in starving *P. putida* cells. As described above, the spectrum of the Phe^+ mutations changed during prolonged starvation: the proportion of 2- to 3-bp deletions among the promoter-creating mutations increased and the number of transversions declined (Table 2). This indicated changes in expression of some mutagenic pathway in *P. putida* cells during starvation. Stationary-phase sigma factor σ^S (RpoS) is the main sigma factor activating gene expression in stationary phase or otherwise stressed bacterial cells (reviewed in reference 28).

To study the possible effect of RpoS on stationary-phase mutations in *P. putida*, we characterized the spectrum of Phe^+ mutations in starving cultures of the *P. putida rpoS*-defective mutant PKS54, which is a derivative of *P. putida* wild-type strain PaW85 (47). Ilves et al. have previously shown that

TABLE 4. Effect of number of Phe^+ colonies on selective plates on further accumulation of mutants in wild-type and MutY-defective *P. putida* strains[a]

Day	Mean no. of Phe^+ colonies ± SD at initial inocolum:					
	5×10^8 cells		5×10^7 cells		5×10^6 cells	
	Wild type	*mutY*	Wild type	*mutY*	Wild type	*mutY*
2	2.0 ± 1.7	260 ± 108	0	30 ± 13	0	3.0 ± 1.8
3	2.3 ± 1.7	423 ± 33	0	92 ± 13	0	10 ± 2.6
4	9.3 ± 4.5	108 ± 30	1.8 ± 0.5	183 ± 29	0	23 ± 2.6
5	19 ± 9.8	37 ± 15	0.8 ± 0.9	113 ± 43	0	25 ± 5.1
6	41 ± 20	8.3 ± 2.5	1.8 ± 2.4	22 ± 11	0	60 ± 6.5
7	39 ± 21	5.2 ± 1.9	2.8 ± 2.0	15 ± 6.7	0	72 ± 9.6

[a] Different amounts of pEST1414-carrying cells (5×10^8, 5×10^7, or 5×10^6) were plated with a constant number (10^9) of scavenger cells onto phenol minimal plates. Results of four separate platings are shown.

TABLE 5. Spectrum of Phe$^+$ mutations occurring in starving cultures of the RpoS-defective *P. putida* strain

Days of starvation	No. of mutants analyzed	No. (%) of mutants with:			
		C-to-A transversion	G-to-T transversion	Deletions (7 to 80 nt)	Undefined mutations
4	74	46 (62)	23 (31)	3 (4)	2 (3)
6 and 7	71	26 (36)	22 (31)	2 (3)	21 (30)

transposition of Tn*4652* into the reporter plasmid pEST1332 is suppressed in the *P. putida* RpoS-defective strain (30). Therefore, we expected to see some decrease in the frequency of Phe$^+$ mutations in this background.

Indeed, in comparison with the wild-type strain, the frequency of appearance of Phe$^+$ mutants was lower in the RpoS-defective strain (Fig. 1). During the first 5 days of starvation, this was even lower than the theoretical frequency of appearance of mutants in the wild-type strain if Tn*4652* insertions were subtracted (Fig. 1). Because, during the first 5 days of starvation, the RpoS-defective strain survives as well as the wild-type strain (30), the lower rate of accumulation of Phe$^+$ mutants in strain PKS54 than in PaW85 cannot be explained by lower viability of the RpoS mutant. Surprisingly, on days 6 and 7 we observed an increase in the number of Phe$^+$ mutants that emerged per plate in PKS54 compared to that in the wild-type strain.

Analysis of the mutants revealed that the higher rate of accumulation of Phe$^+$ mutants in the RpoS-defective strain was caused by activation of transposition of IS*1411* (Fig. 1). The proportion of IS*1411*-linked Phe$^+$ mutants increased during starvation from 10% on day 3 to 75% on day 7. IS*1411* is located just downstream of the *pheBA* genes in reporter plasmid pEST1414 (31). IS*1411* can insert upstream of the promoterless *pheBA* operon and activate transcription of these genes due to the presence of outward-directed promoters at the left end of IS*1411*. However, intramolecular transposition of IS*1411* in the wild-type strain PaW85 is rare, being lower than 1% of the events that activate the reporter genes in pEST1414 (31). We did not detect transposition of Tn*4652* into the reporter plasmid pEST1414 in the RpoS-defective *P. putida* strain PKS54.

As the next step of our investigations, we concentrated on characterization of the mutants that emerged independently of IS*1411* insertions. The Phe$^+$ colonies that appeared on selective plates on day 4 and days 6 to 7 were subjected to sequence analysis. The data presented in Table 5 show that the occurrence of 2- to 3-bp deletions was positively controlled by RpoS. Only a few deletions in range of 7 to 80 bp were observed in starving cells of the RpoS mutant, but no 2- to 3-bp deletions were recorded. In addition to small deletions, 2- to 3-bp insertions were also absent in the RpoS mutant.

We controlled whether the promoters created by the 2- to 3-bp deletions could express the *pheBA* genes in *P. putida* RpoS-defective strain at the level enabling growth of bacteria on phenol minimal plates. We transformed the RpoS-defective strain PKS54 with deletant plasmids that were isolated from Phe$^+$ mutants of the wild-type cells. In all cases studied, as in the wild-type strain, the Phe$^+$ transformants of the RpoS-defective strain emerged on phenol minimal plates on day 2 after plating.

DISCUSSION

The test system for the selection of mutants based on creation of functional promoter sequences for the transcriptional activation of the *pheBA* phenol degradation genes in *P. putida* enables us to study different types of mutations and transposition of mobile DNA elements. Based on the analysis of the mutants characterized in this study and previously (32), the sequence upstream of the *pheBA* genes contains many potential −35 and −10 hexamers for promoter creation (Fig. 2). Most of these −35 and −10 elements are located too far from each other, but the distance between the promoter elements can be optimized by deletions.

The length of the deletions between six different −35 hexamers and four different −10 hexamers that created a functional promoter varied from 2 to 80 bp. In some cases, the length of the spacer between the −35 sequence TTGCAC and −10 sequence CATAAT was extended from a nonoptimal 15 bp to the optimal distance (17 or 18 bp) due to 2-bp or 3-bp insertions. Additionally, two point mutations changed two originally nonfunctional −10 hexamers located at the right distance from the −35 promoter elements to functional −10 sequences. One particular C-to-A transversion 96 nucleotides upstream from the ATG codon of the *pheB* gene changed the original sequence T**C**GACT to T**A**GACT, which is more similar to the −10 hexamer consensus sequence TATAAT. Another G-to-T transversion 200 nucleotides upstream from the *pheB* gene converted the potential −10 sequence TAATC**G** to TAATC**T**.

The nature of the mutations creating promoters for the transcriptional activation of the *pheBA* genes in *P. putida* is influenced by physiological state of the bacteria. Data presented previously (32) and herein indicate that the mutation generation process in starving cells is different from that in growing bacteria. Various deletions, mostly larger than 2 to 3 bp, were highly represented in mutants emerging among growing bacteria, but they rarely appeared in starving cultures. Moreover, differences were also revealed between early-arising and late-arising stationary-phase mutations. The most dominant mutation type in starving cells, the C-to-A transversion, was characteristic of the mutants that appeared on selective plates on days 3 to 5 after plating. Later (days 6 and 7) we observed an increase in the frequency of 2- to 3-bp deletions and a decrease in the frequency of the C-to-A transversion (Table 2).

The specific spectrum of mutations depends on a complex array of factors, including replication errors, lesions in DNA, and changes due to repair. There are several hints about DNA repair insufficiency in starving cells of *E. coli* (reviewed in reference 21). The oxidation of guanine to 7,8-dihydro-8-oxoguanine is one of the most common types of DNA damage in cells. Oxidative DNA damage accumulation within bacteria is a major contributor to the generation of stationary-phase mutations in bacteria (10). MutY is part of a complex DNA repair network that reduces the mutagenic effects of 7,8-dihydro-8-oxoguanine. MutY is a glycosylase which removes adenine from A:7,8-dihydro-8-oxoguanine mispairs in duplex DNA

(42), and failure to perform this function in *mutY* strains leads to a significant increase in G:C to T:A transversions (45).

Recently, it was found that artificial overexpression of methyl-directed DNA mismatch repair enzyme MutS in the *E. coli* CC104 tester strain also significantly decreased G:C to T:A transversions (68). We have constructed a MutY-defective strain of *P. putida*. Study of the spectrum of promoter-creating mutations in the *P. putida* MutY-defective strain revealed a tight connection between expression of MutY and the frequency of occurrence of two-base substitutions and C-to-A and G-to-T transversions. The lack of 7,8-dihydro-8-oxoguanine repair enzyme MutY in *P. putida* cells gave rise to this type of mutation in both growing and starving cells. The frequency of promoter-creating mutations in mismatch repair enzyme MutS-defective *P. putida* also constructed in our laboratory increased only in starving cultures, but most of these mutations occurred outside the sequencing window (data not shown).

Based on these data, we suppose that the lower efficiency of the 7,8-dihydro-8-oxoguanine repair pathway in starving *P. putida* might be the reason for the high frequency of occurrence of C-to-A transversion at one particular site upstream of the *pheB* gene in early-arising stationary-phase mutants. However, some other types of Phe^+ mutations (located outside of the sequencing window) occurred in starving populations of *P. putida* PaW85 *mutY*::*tet* cells in addition to C-to-A and G-to-T transversions, and the frequency of these undefined mutations increased during starvation.

Bridges and Timms (11) reported that when *trpA mutY* cells of *E. coli* were held under tryptophan starvation conditions, the tryptophan-independent mutations that arose included small in-frame deletions in addition to transversions. Such deletions were also found in starved bacteria defective in the mismatch repair system. The deletant mutants appeared later, and the delayed appearance was caused by slower growth of these mutants compared to the transversion mutants (11). In the case of the appearance of the Phe^+ mutants during starvation, the shift from transversions to 2- to 3-bp deletions cannot be accounted for by slower growth of the deletant mutants on phenol minimal plates. Also, we did not observe an increase in the rate of occurrence of the 2- to 3-bp deletions in either the *P. putida* mismatch repair- or 7,8-dihydro-8-oxoguanine repair pathway-defective strain.

We have found that the formation of small deletions is a regulated process that needs stationary-phase sigma factor RpoS. These deletions (and also small 2- to 3-bp insertions) were absent in the RpoS-defective *P. putida* strain. The results obtained by us indicate that some mutagenic pathway responsible for the creation of small deletions and insertions must be positively controlled by the stationary-phase sigma factor in *P. putida*. The effect of this pathway becomes more evident in cells that have been starving for some days. It is obvious that there should be a balance among the various processes involved in mutagenesis, DNA damage, various types of DNA replication and DNA repair, metabolic activation, detoxification, and, perhaps, also in the environment of cells.

The effect of environment on mutation spectra has recently been demonstrated by studying compensatory mutations to antibiotic resistance (7). One could speculate that during prolonged starvation, the physiological parameters of the bacterial cell and also the environment where bacteria are starving might differ from those at the beginning of starvation. There are several reports indicating that changes in gene expression take place even in late stationary phase (23, 24, 43, 63; reviewed in reference 36). Therefore, these changes might affect DNA metabolism functions in starving cells.

Evidence for the involvement of error-prone polymerases in stationary-phase mutations has been demonstrated in several studies (6, 13, 38, 40, 64). Recent studies indicate that *E. coli* DNA polymerases act coordinately with their associated accessory factors in the context of DNA replication, repair, and recombination (60). Most prokaryotes carry homologues of the error-prone DinB/UmuC/Rad30/Rev1 superfamily polymerases (39). Thus, drawing parallels with mutagenesis in *E. coli*, the DNA polymerase switch is one possible mechanism for changes in the spectrum of promoter-creating mutations during starvation of *P. putida*. A gene homologous to *E. coli dinB* is also present in the genome of *P. putida*, but regulation of the expression of the mutagenic polymerases in this organism has not been unexplored.

It seems very likely that increased activity of mutagenic DNA polymerase in cells that have already been starved for some days could explain changes in the spectrum of Phe^+ mutations. However, one could also argue that different extents of stress-induced DNA protection at different stages of starvation would affect the outcome of mutagenesis. Under conditions of either oxidative or nutritional stress, *E. coli* produces high level of the nonspecific DNA-binding protein Dps (DNA-binding protein from starved cells), which efficiently protects DNA against oxidative agents (2, 3, 37). *dps* mutants exhibit an increased level of G:C to T:A transversions (37). Dps was discovered as a protein synthesized at a high level after *E. coli* had been in stationary phase for over 3 days (2). An increase in Dps level after prolonged cultivation of *E. coli* cells was also observed by Azam et al. (4).

Close Dps homologs have been identified in distantly related bacteria (see the references in reference 66), which implies that this mode of DNA protection might be general and crucial. DNA sequence database analysis indicates that a *dps* homolog is also present in *Pseudomonas* spp. (data not shown). Thus, drawing parallels with Dps function in *E. coli*, we can speculate that the effect of Dps on protecting DNA against oxidative damage should become obvious only after prolonged starvation of *P. putida*. Phe^+ cells of *P. putida* mixed with Phe^- cells need 2 days to form visible colonies on phenol minimal plate (32). Therefore, the mutants which were picked from selective plates earlier (on days 3 to 5; they appeared mostly due to transversions) represent events that were happening at the beginning of starvation, when DNA is probably less protected. Later, on days 6 to 7, when DNA is becoming more resistant to 7,8-dihydro-8-oxoguanine mutagenesis, the proportion of the other type of mutations, not dependent on the 7,8-dihydro-8-oxoguanine repair system (e.g., small deletions), increases. This possible change, in addition to hypothetical induction of mutagenic DNA polymerase, would also explain why small deletions become more abundant during prolonged starvation.

Both transposon Tn*4652* and IS*1411* can activate transcription of promoterless *pheBA* genes in *P. putida* (30, 31, 32). The movement of transposable DNA elements is mediated by functions encoded by the element and by its host (34). Transposons

can usually insert into many different sites of the genome, which renders the movement of a transposon potentially deleterious for host gene expression. Therefore, the movement of mobile DNA elements is usually strongly downregulated. The advantageousness of transposition sometimes appears only in extreme situations, when transposition permits new traits to evolve (56).

Our studies of transposition of Tn*4652* and IS*1411* also indicate that DNA rearrangement activities under stress are controlled by many cellular regulatory functions which might be affected by how long bacteria have been starved. Ilves et al. found that the insertion of transposon Tn*4652* is an exclusively stationary-phase-specific event regulated by stationary-phase sigma factor σ^S (30). Control of the timing of genetic changes became evident in transposition of Tn*4652*; Kasak et al. have demonstrated that the maximum rate of transposition frequency of this DNA element is achieved after a few days of starvation of *P. putida* cells (32). The results presented in the current report demonstrate that activation of the transposition of IS*1411* (in contrast to the movement of Tn*4652*) seems to be negatively controlled by RpoS, which means that expression of some factor(s) downregulating IS*1411* transposition activity requires RpoS. Interestingly, the transposition of IS*1411* increases remarkably with time of starvation. This hints that activation of IS*1411* needs some late-starvation signal.

To summarize the results of studies on stationary-phase mutations presented in this report, we suggest that mutation processes in cells that have been starved for short periods are not entirely compatible with prolonged starvation, at least in *P. putida*. In nature, survival is the normal mode for most microorganisms, and growth occurs only occasionally. Prolonged starvation is a condition in which bacteria undergo rapid evolution by natural selection (61, 62, 67; see also the references in references 17 and 53). Thus, to understand the mechanisms of molecular evolution in microorganisms, more attention should be paid to stationary-phase mutations occurring during prolonged starvation.

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REFERENCES

1. **Adams, M. H.** 1959. Bacteriophages, p. 445–447. Interscience Publishers Inc., New York, N.Y.
2. **Almiron, M., A. J. Link, D. Furlong, and R. Kolter.** 1992. A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. Genes Dev. **6:**2646–2654.
3. **Altuvia, S., M. Almiron, G. Huisman, R. Kolter, and G. Storz.** 1994. The *dps* promoter is activated by OxyR during growth by IHF and σ^S in stationary-phase. Mol. Microbiol. **13:**265–272.
4. **Azam, T. A., A. Iwata, A. Nishimura, S. Ueda, and A. Ishihama.** 1999. Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. J. Bacteriol. **181:**6361–6370.
5. **Bayley, S. A., C. J. Duggleby, M. J. Worsey, P. A. Williams, K. G. Hardy, and P. Broda.** 1977. Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. **154:**203–204.
6. **Bhamre, S., B. B. Gadea, C. A. Koyama, S. J. White, and R. G. Fowler.** 2001. An aerobic *recA*-, *umuC*-dependent pathway of spontaneous base-pair substitution mutagenesis in *Escherichia coli*. Mutat. Res. **473:**229–247.
7. **Björkman, J., I. Nagaev, O. G. Berg, D. Hughes, and D. I. Andersson.** 2000. Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. Science **287:**1479–1482.
8. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
9. **Bridges, B. A.** 1997. DNA turnover and mutation in resting cells. BioEssays **19:**347–352.
10. **Bridges, B. A.** 1998. The role of DNA damage in stationary-phase ("adaptive") mutation. Mutat. Res. **408:**1–9.
11. **Bridges, B. A., and A. R. Timms.** 1997. Mutation in *Escherichia coli* under starvation conditions: a new pathway leading to small deletions in strains defective in mismatch correction. EMBO J. **16:**3349–3356.
12. **Bridges, B. A., M. Sekiguchi, and T. Tajiri.** 1996. Effect of *mutY* and *mutM*/*fpg-1* mutations on starvation-associated mutation in *Escherichia coli*: implications for the role of 7,8-dihydro-8-oxoguanine. Mol. Gen. Genet. **251:**352–357.
13. **Bull, H. J., M.-J. Lombardo, and S. M. Rosenberg.** 2001. Stationary-phase mutation in the bacterial chromosome: recombination protein and DNA polymerase IV dependence. Proc. Natl. Acad. Sci. USA **98:**8334–8341.
14. **Capy, P., G. Gasperi, C. Biemont, and C. Bazin.** 2000. Stress and transposable elements: coevolution or useful parasites? Heredity **85:**101–106.
15. **Carter, P., H. Bedouelle, and G. Winter.** 1985. Improved oligonucleotide site-directed mutagenesis with M13 vectors. Nucleic Acids Res. **13:**4431–4443.
16. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
17. **Finkel, S. E., E. R. Zinser, and R. Kolter.** 2000. Long-term survival and evolution in the stationary phase, p. 231–238. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. American Society for Microbiology, Washington, D.C.
18. **Foster, P. L.** 1997. Nonadaptive mutations occur on the F′ episome during adaptive mutation conditions in *Escherichia coli*. J. Bacteriol. **179:**1550–1554.
19. **Foster, P. L.** 1998. Adaptive mutation: has the unicorn landed? Genetics **148:**1453–1459.
20. **Foster, P. L.** 1999. Are adaptive mutations due to a decline in mismatch repair? The evidence is lacking. Mutat. Res. **436:**179–184.
21. **Foster, P. L.** 1999. Mechanisms of stationary-phase mutation: a decade of adaptive mutation. Annu. Rev. Genet. **33:**57–88.
22. **Foster, P. L., and J. M. Trimarchi.** 1994. Adaptive reversion of a frameshift mutation in *Escherichia coli* by simple base deletions in homopolymeric runs. Science **265:**407–409.
23. **Giskov, M., L. Eberl, and S. Molin.** 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: two-dimensional electrophoretic analysis of starvation- and stress-induced proteins. J. Bacteriol. **176:**4816–4824.
24. **Giskov, M., L. Eberl, S. Møller, L. K. Poulsen, and S. Molin.** 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. J. Bacteriol. **176:**7–14.
25. **Hanahan, D.** 1983. Studies on the transformation of *E. coli* with plasmids. J. Mol. Biol. **166:**577–580.
26. **Harris, R. S., G. Feng, K. J. Ross, R. Sidhu, C. Thulin, S. Longerich, S. K. Szigety, P. J. Hastings, M. E. Winkler, and S. M. Rosenberg.** 1999. Mismatch repair is diminished during stationary-phase mutation. Mutat. Res. **437:**51–60.
27. **Hegeman, G. D.** 1966. Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of the enzymes by wild type. J. Bacteriol. **91:**1140–1154.
28. **Hengge-Aronis, R.** 2000. The general stress response in *E. coli*, p. 161–177. *In* G. Storz, and R. Hengge-Aronis (ed.), Bacterial stress responses. American Society for Microbiology, Washington, D.C.
29. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
30. **Ilves, H., R. Hõrak, and M. Kivisaar.** 2001. Involvement of σ^S in starvation-induced transposition of *Pseudomonas putida* transposon Tn*4652*. J. Bacteriol. **183:**5445–5448.
31. **Kallastu, A., R. Hõrak, and M. Kivisaar.** 1998. Identification and characterization of IS*1411*, a new insertion sequence which causes transcriptional activation of the phenol degradation genes in *Pseudomonas putida*. J. Bacteriol. **180:**5306–5312.
32. **Kasak, L., R. Hõrak, and M. Kivisaar.** 1997. Promoter-creating mutations in *Pseudomonas putida*: a model system for the study of mutation in starving bacteria. Proc. Natl. Acad. Sci. USA **94:**3134–3139.
33. **Kivisaar, M., L. Kasak, and A. Nurk.** 1991. Sequence of the plasmid-encoded catechol 1,2-dioxygenase-expressing gene, *pheB*, of phenol-degrading *Pseudomonas* sp. strain EST 1001. Gene **98:**15–20.
34. **Kleckner, N.** 1990. Regulation of transposition in bacteria. Annu. Rev. Cell Biol. **6:**297–327.
35. **Kurusu, Y., T. Narita, M. Suzuki, and T. Watanabe.** 2000. Genetic analysis of an incomplete *mutS* gene from *Pseudomonas putida*. J. Bacteriol. **182:**5278–5279.

36. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor σ^S (KatF) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.
37. **Martinez, A., and R. Kolter.** 1999. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. J. Bacteriol. **179:**5188–5194.
38. **McBeth, D. L, and B. Hauer.** 1996. Increased mutagenesis mediated by cloned plasmid CAM-OCT genes: potential for expanding substrate ranges of *Pseudomonas* spp. Appl Environ. Microbiol. **62:**3538–3540.
39. **McKenzie, G. J., and S. M. Rosenberg.** 2001. Adaptive mutations, mutator DNA polymerases and genetic change strategies of pathogens. Curr. Opin. Microbiol. **4:**586–594.
40. **McKenzie, G. J., P. L. Lee, M.-J. Lombardo, P. J. Hastings, and S. M. Rosenberg.** 2001. SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. Mol. Cell **7:**571–579.
41. **Michaels, M. L., and J. H. Miller.** 1992. The 7,8-dihydro-8-oxoguanine system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). J. Bacteriol. **174:**6321–6325.
42. **Michaels, M. L., C. Cruz, A. P. Grollman, and J. H. Miller.** 1992. Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. Proc. Natl. Acad. Sci. USA **89:**7022–7025.
43. **Miksch, G., and P. Dobrowolski.** 1995. Growth phase-dependent induction of stationary-phase promoters of *Escherichia coli* in different gram-negative bacteria. J. Bacteriol. **177:**5374–5378.
44. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Nghiem, Y., M. Cabrera, C. G. Cupples, and J. H. Miller.** 1988. The *mutY* gene: a mutator locus in *Escherichia coli* that generates G:C-T:A transversions. Proc. Natl. Acad. Sci. USA **85:**2709–2713.
46. **Nurk, A., A. Tamm, R. Hõrak, and M. Kivisaar.** 1993. In-vivo-generated fusion promoters in *Pseudomonas putida*. Gene **127:**23–29.
47. **Ojangu, E.-L., A. Tover, R. Teras, and M. Kivisaar.** 2000. Effects of combination of different −10 hexamers and downstream sequences on stationary-phase-specific sigma factor σ^S-dependent transcription in *Pseudomonas putida*. J. Bacteriol. **182:**6707–6713.
48. **Oliver, A., F. Baquero, and J. Blázquez.** 2002. The mismatch repair system (*mutS, mutL and uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. Mol. Microbiol. **43:**1641–1650.
49. **Oliver, A., R. Cantón, P. Campo, F. Baquero, and J. Blázquez.** 2000. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science **288:**1251–1253.
50. **Pavel, H., M. Forsman, and V. Shingler.** 1994. An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of *Pseudomonas* sp. strain CF600 on para-substituted methylphenols. J. Bacteriol. **176:**7550–7557.
51. **Powell, S. C., and R. M. Wartell.** 2001. Different characteristics distinguish early versus late arising adaptive mutations in *Escherichia coli* FC40. Mutat. Res. **473:**219–228.
52. **Prival, M. J., and T. A. Cebula.** 1992. Sequence analysis of mutations arising during prolonged starvation of *Salmonella typhimurium*. Genetics **132:**303–310.
53. **Rosche, W. A., and P. L. Foster.** 2000. Mutation under stress: adaptive mutation in *Escherichia coli*, p. 239–248. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. American Society for Microbiology, Washington, D.C.
54. **Rosenberg, S. M., C. Thulin, and R. S. Harris.** 1998. Transient and heritable mutators in adaptive evolution in the lab and in nature. Genetics **148:**1559–1566.
55. **Rosenberg, S. M., S. Longerich, P. Gee, and R. S. Harris.** 1994. Adaptive mutation by deletions in small mononucleotide repeats. Science **265:**405–407.
56. **Shapiro, J. A.** 1997. Genome organization, natural genetic engineering and adaptive mutation. Trends Genet. **13:**98–104.
57. **Sharma, R. C., and R. T. Schimke.** 1996. Preparation of electro-competent *E. coli* with salt-free growth medium. BioTechniques **20:**42–44.
58. **Sniegowski, P. D., P. J. Gerrish, and R. E. Lenski.** 1997. Evolution of high mutation rates in experimental populations of *E. coli*. Nature **387:**703–705.
59. **Spiers, A. J., A. Buckling, and P. B. Rainey.** 2000. The causes of *Pseudomonas* diversity. Microbiology **146:**2345–2350.
60. **Sutton, M. D., and G. C. Walker.** 2001. Managing DNA polymerases: coordinating DNA replication, DNA repair, and DNA recombination. Proc. Natl. Acad. Sci. USA **98:**8342–8349.
61. **Taddei, F., I. Matic, and M. Radman.** 1995. cAMP-dependent SOS induction and mutagenesis in resting bacterial populations. Proc. Natl. Acad. Sci. USA **92:**11736–11740.
62. **Taddei, F., J. A. Halliday, I. Matic, and M. Radman.** 1997. Genetic analysis of mutagenesis in aging *Escherichia coli* colonies. Mol. Gen. Genet. **256:**277–281.
63. **Talukder, A. A., S. Yanai, T. Nitta, A. Kato, and M. Yamada.** 1996. RpoS-dependent regulation of genes expressed at late stationary-phase in *Escherichia coli*. FEBS Lett. **386:**177–180.
64. **Timms, A. R., W. Muriel, and B. A. Bridges.** 1999. UmuD,C-dependent pathway for spontaneous G:C to C:G transversions in stationary-phase *Escherichia coli mutY*. Mutat. Res. **434:**77–80.
65. **Torkelson, J., R. S. Harris, M.-J. Lombardo, J. Nagendran, C. Thulin, and S. M. Rosenberg.** 1997. Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation. EMBO J. **16:**3303–3311.
66. **Wolf, S. G., D. Frenkiel, T. Arad, S. E. Finkel, R. Kolter, and A. Minsky.** 1999. DNA protection by stress-induced biocrystallization. Nature **400:**83–85.
67. **Zambrano, M. M., D. A. Siegele, M. Almiron, A. Tormo, and R. Kolter.** 1993. Microbial competition: *Escherichia coli* mutants that take over stationary-phase cultures. Science **259:**1757–1760.
68. **Zhao, J., and M. E. Winkler.** 2000. Reduction of GC → TA transversion mutation by overexpression of MutS in *Escherichia coli* K-12. J. Bacteriol. **182:**5025–5028.

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Oxidative DNA Damage Defense Systems in Avoidance of Stationary-Phase Mutagenesis in *Pseudomonas putida*[▽]

Signe Saumaa, Andres Tover, Mariliis Tark, Radi Tegova, and Maia Kivisaar*

Department of Genetics, Institute of Molecular and Cell Biology, Tartu University and Estonian Biocentre, 51010 Tartu, Estonia

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Oxidative damage of DNA is a source of mutation in living cells. Although all organisms have evolved mechanisms of defense against oxidative damage, little is known about these mechanisms in nonenteric bacteria, including pseudomonads. Here we have studied the involvement of oxidized guanine (GO) repair enzymes and DNA-protecting enzyme Dps in the avoidance of mutations in starving *Pseudomonas putida*. Additionally, we examined possible connections between the oxidative damage of DNA and involvement of the error-prone DNA polymerase (Pol)V homologue RulAB in stationary-phase mutagenesis in *P. putida*. Our results demonstrated that the GO repair enzymes MutY, MutM, and MutT are involved in the prevention of base substitution mutations in carbon-starved *P. putida*. Interestingly, the antimutator effect of MutT was dependent on the growth phase of bacteria. Although the lack of MutT caused a strong mutator phenotype under carbon starvation conditions for bacteria, only a twofold increased effect on the frequency of mutations was observed for growing bacteria. This indicates that MutT has a backup system which efficiently complements the absence of this enzyme in actively growing cells. The knockout of MutM affected only the spectrum of mutations but did not change mutation frequency. Dps is known to protect DNA from oxidative damage. We found that *dps*-defective *P. putida* cells were more sensitive to sudden exposure to hydrogen peroxide than wild-type cells. At the same time, the absence of Dps did not affect the accumulation of mutations in populations of starved bacteria. Thus, it is possible that the protective role of Dps becomes essential for genome integrity only when bacteria are exposed to exogenous agents that lead to oxidative DNA damage but not under physiological conditions. Introduction of the Y family DNA polymerase PolV homologue *rulAB* into *P. putida* increased the proportion of A-to-C and A-to-G base substitutions among mutations, which occurred under starvation conditions. Since PolV is known to perform translesion synthesis past damaged bases in DNA (e.g., some oxidized forms of adenine), our results may imply that adenine oxidation products are also an important source of mutation in starving bacteria.

Bacteria spend most of the time under starvation conditions, when their growth is inhibited due to a shortage of energy. Still, populations of stationary-phase bacteria undergo rapid evolution because of mutations. Mutagenesis occurring in resting cells is called adaptive mutation or stationary-phase mutation (22, 23). Bacteria have several stress responses (including induction of the error-prone DNA polymerases carrying out translesion synthesis) that provide ways in which mutation rates can be increased in stationary-phase cells (for reviews, see references 24, 47, and 64). Oxidative DNA damage is also an important source of mutagenesis. It is known that the formation of 7,8-dihydro-8-oxo-2′-deoxyguanine (8-oxoG or GO) can give rise to stationary-phase mutations in *Escherichia coli* (13, 14). Also, products of the oxidative damage of adenine have been shown to be mutagenic (36). However, so far, these oxidation products have received less attention.

Bacteria have evolved different ways to protect their DNA from oxidative damage. For example, stationary-phase-specific protein Dps protects *E. coli* against multiple stresses, including the avoidance of oxidative damage of DNA by exogenous agents (2, 46, 56). The protection is promoted by the formation of a crystalline structures composed of DNA and Dps (26, 78). Dps homologues are present in many distantly related bacteria, indicating that DNA protection by Dps may be crucial and widespread in prokaryotes. Martinez and Kolter (46) have demonstrated that in the presence of exogenously added hydrogen peroxide, the lack of Dps resulted in a 10-fold increase in the frequency of mutations in *E. coli*. However, whether the presence of Dps could influence the frequency of mutations under physiological conditions in bacteria is still unexplored.

In order to mitigate the mutagenic effect of 8-oxoG, bacteria have developed an oxidized guanine (GO) repair system (52, 54). Oxidatively damaged guanine is removed by MutM glycosylase. If MutM fails to remove 8-oxoG adducts before DNA replication, translesion synthesis can be inaccurate, leading to the misincorporation of A opposite the 8-oxoG adduct (53, 69). Thus, another glycosylase, MutY, removes adenine from A · (8-oxoG) and from A · G mispairings (54). Defective *mutM* and *mutY* alleles both result in enhanced production of G · C-to-T · A transversions (17, 59). The spontaneous mutation frequency is elevated more than 10-fold in MutM-defective *E. coli* (17, 51) and 20- to 100-fold in MutY-defective bacteria (59). MutT pyrophosphohydrolase hydrolyzes 8-oxodGTP to 8-oxodGMP and pyrophosphate to prevent its use as a substrate by replicative DNA polymerase III (PolIII) (45) or by Y family DNA polymerases (70, 80). Inactivation of MutT in *E. coli* leads to a specific increase in A · T-to-C · G transversions, resulting from a misincorporation of 8-oxodGTP opposite template A (45, 81).

* Corresponding author. Mailing address: Department of Genetics, Institute of Molecular and Cell Biology, Tartu University and Estonian Biocentre, 23 Riia Street, 51010 Tartu, Estonia. Phone: 372-7-375036. Fax: 372-7-420286. E-mail: maiak@ebc.ee.

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It has been demonstrated that alterations in the genes of DNA mismatch repair (MMR) and in the *mutY* gene were responsible for increased mutation frequency in mutator *Pseudomonas aeruginosa* strains from cystic fibrosis patients, whereas no mutator strains were identified with the inactivation of the *mutM* or *mutT* gene (60, 61). Additionally, a *mutY*-defective derivative of *Pseudomonas fluorescens* showed a strongly enhanced competitive root-tip-colonizing phenotype through accelerated evolution (20). The fact that the absence of MutY increases the mutation frequency of *Pseudomonas* species has also been demonstrated in our work with *P. putida* (67). *P. aeruginosa mutY*, *mutM*, and *mutT* genes were shown to complement those of *E. coli* GO repair-deficient strains, but no knockouts of these genes were constructed in *P. aeruginosa* (61). Thus, it is still unclear whether, besides MutY, the absence of MutM or MutT activity could also cause a mutator phenotype in *Pseudomonas* species.

8-oxoG is not generally considered to be a replication-blocking lesion (10). On the other hand, in vitro studies have revealed that 8-oxoG is highly susceptible to further oxidation and yields a variety of additional products, some of which block DNA replication (reviewed in reference 58). Translesion synthesis DNA polymerases PolII, PolIV, and PolV allow replication to progress in the presence of DNA lesions that are strongly inhibitory to the replicative DNA polymerase PolIII (28). Translesion synthesis by PolIV and PolV is error prone (28, 73) and has been shown to participate in stationary-phase mutagenesis (8, 16, 49, 72, 75, 82). Recently published studies by Neeley et al. (57) suggest a major role for PolV and minor roles for PolII and PolIV in the mechanism of guanine oxidation mutagenesis in *E. coli* strains containing engineered M13 viral genomes with guanine oxidation products.

P. putida, like many other bacterial species, does not carry PolV genes in its chromosome. Instead, DNA polymerase PolV homologues are frequently encoded by naturally occurring conjugative plasmids in these bacteria (e.g., see reference 74 and references therein). Our previous studies have revealed that the PolV homologue RulAB from TOL plasmid pWW0 increases the evolutionary fitness of starving *P. putida* bacteria (74). This raises the question of whether this polymerase could be involved in stationary-phase mutagenesis via a mechanism which is dependent on oxidative damage of DNA.

Here we have focused our studies on mechanisms of mutagenesis caused by oxidative damage with *P. putida.* We have investigated the role of MutY, MutM, and MutT homologues in DNA repair in this organism and the roles of Dps in the protection of DNA and in avoidance of mutations. We have also examined the possible relationship between RulAB-dependent mutagenesis and oxidative damage of DNA in starving *P. putida* organisms. Our results demonstrated that MutT is involved in the avoidance of mutations in starving populations of *P. putida*, whereas the absence of this enzyme has only a slight effect on the mutation frequency in growing bacteria. The phenotypic effect of the absence of MutM appeared in changes in the spectrum of stationary-phase mutations but not in the mutation frequency. Additionally, our results implied that although Dps protected *P. putida* cells against sudden exposure to the oxidizing agent hydrogen peroxide, this protein had no role in the avoidance of stationary-phase mutations under physiological conditions of bacteria. Introduction of the translesion synthesis DNA polymerase PolV genes *rulAB* from TOL plasmid pWW0 into the *P. putida* chromosome, which originally lacks PolV genes, resulted in an increased frequency of A-to-G and A-to-C base substitutions. These data hinted that in addition to the 8-oxoG lesion, damage of adenine may also be an important endogenously produced mutagen in starved bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. Complete media used were Luria-Bertani (LB) medium (55) and minimal medium M9 (1). Solid medium contained 1.5% Difco agar. Casamino Acids (CAA) and glucose were added to the minimal medium at final concentrations of 0.4% and 0.2%, respectively. Phenol-minimal plates contained 2.5 mM phenol (Phe) as the sole carbon and energy source. Antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin (Km), 50 μg/ml; tetracycline, 80 μg/ml; rifampin (Rif), 100 μg/ml; and carbenicillin, 1,500 to 3,000 μg/ml. *E. coli* was incubated at 37°C and *P. putida* at 30°C. *E. coli* and *P. putida* were electrotransformed as described by Sharma and Schimke (68). *E. coli* strains DH5α (Invitrogen) and CC118 λpir (32) were used for the DNA cloning procedures, and HB101 (12) was used as a host for helper plasmid pRK2013 (21), necessary for mobilization of nonconjugative plasmids.

Construction of *P. putida* GO repair-deficient strains. The *mutM* (PP5125) and *mutT* (PP1348) sequences of *P. putida* KT2440 were obtained from the Institute for Genomic Research website (http://www.tigr.org). These genes were amplified by PCR from genomic DNA of *P. putida* strain PaW85 (6) (this strain is isogenic to KT2440). Thereafter, the internal sequences of the amplified genes were replaced with antibiotic resistance marker genes and DNA fragments carrying an antibiotic resistance determinant, and sequences from 5′ and 3′ ends of particular *mut* genes were inserted into plasmid pGP704 L (62), not able to replicate in hosts other than *E. coli* CC118 λpir. The wild-type sequences of the *mutM* and *mutT* genes present in the chromosome of *P. putida* strain PaW85 were replaced with the interrupted ones by homologous recombination. Derivatives of the plasmid pGP704 L carrying replacement cassettes were conjugatively transferred into *P. putida* PaW85 by using helper plasmid pRK2013 (21). The integration of whole-delivery plasmid into a target site was excluded by testing transconjugants for their resistance to carbenicillin (only those unable to grow in the presence of 3,000 μg/ml carbenicillin were considered to be true recombinants, generated as a result of double recombination events). PaW85 derivatives with desired gene knockouts were verified by PCR analysis.

The 810-bp *mutM* gene was amplified by PCR from the genomic DNA of *P. putida* strain PaW85. The primers mutMylem (5′-CATTGGATTGTCGGAAT ACGA-3′), complementary to the nucleotide sequence −70 to −50 relative to the ATG initiator codon of the *mutM* gene, and mutMalum (5′-CGGATCACT GGCAAGGGCT-3′), complementary to the nucleotide sequence +111 to +93 downstream of the TGA stop codon of the *mutM* gene, were used for the amplification of the *mutM* gene. The amplified 995-bp DNA fragment containing the *mutM* gene was inserted into the EcoRV-cleaved vector plasmid pBluescript KS(+) to obtain plasmid pKSmutM. The Km^r gene was amplified by PCR from plasmid pUTmini-Tn*5* Km2 by using the primer KmSac, which binds at positions 105 to 126 upstream of the ATG start codon of the Km^r gene and at positions 61 to 62 downstream of the TAA stop codon of the Km^r gene (33). The Ecl136II-cleaved DNA fragment containing the Km^r gene was used to replace the PaeI- and Van91I-generated 525-bp fragment in the *mutM* gene in plasmid pKSmutM. The PaeI ends were blunt ended before the ligation. The Acc65I- and XbaI-generated 1,531-bp Δ*mutM*::*Km* sequence from plasmid pKSΔmutM::Km was inserted into vector plasmid pGP704 L, opened with the same restriction enzymes. The plasmid pGP704ΔmutM::Km was selected in *E. coli* strain CC118 λpir. The interrupted *mutM* gene containing the internal deletion was inserted into the chromosome of *P. putida* PaW85 by homologous recombination. Plasmid pGP704ΔmutM::km, not able to replicate in hosts other than *E. coli* CC118 λpir, was conjugatively transferred into *P. putida* PaW85 by using helper plasmid pRK2013. The PaW85 Δ*mutM*::*km* knockout strain PaWMutM was verified by PCR analysis using primers mutMlookus (5′-ATCTGCGCGTCCTACCGGG-3′), complementary to nucleotide sequence −150 to −132 upstream of the ATG initiator codon of the *mutM* gene, and KmOc (5′-TCGAGCAAGACGTTTCC C-3′), complementary to nucleotide sequence 34 to 16 downstream of the ATG initiator codon of the Km^r gene.

The primers mutTylem (5′-AGTAGCGGCCGTCATTCGCG-3′), complementary to nucleotide sequence +18 to +37 downstream of the ATG initiator

TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Genotype or construction	Source or reference
E. coli		
TG1	*supE hsd* Δ*5 thi* Δ(*lac-proAB*) *F′* (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	18
DH5α	*supE44* Δ*lacU169* (Φ*80 lacZ*Δ*M15*) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Invitrogen
HB101	*subE44 subF58 hsdS3* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	12
CC118 λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (*Am*) *recA1* λ*pir* phage lysogen	32
P. putida		
PaW85	Wild type	6
PaWRulAB	PaW85, carrying the *rulAB* genes from pWW0 in chromosome in *att*Tn*7* site	This study
PaWMutY	PaW85, *mutY*::*tet*	This study
PaWMutM	PaW85, Δ*mutM*::*km*	This study
PaWMutT	PaW85, Δ*mutT*::*km*	This study
PaWDps	PaW85, Δ*dps*::*km*	This study
PaWMutYRulAB	PaWRulAB, *mutY*::*tet*	This study
PaWMutMRulAB	PaWRulAB, Δ*mutM*::*km*	This study
PaWMutTRulAB	PaWRulAB, Δ*mutT*::*km*	This study
Plasmids		
pBluescript KS(+)	Cloning vector (Ap^r)	Stratagene
pUTmini-Tn*5* Km2	Delivery plasmid for mini-Tn*5* Km2 (Ap^r Km^r)	19
pGP704 L	Delivery plasmid for homologous recombination (Ap^r)	62
pRK2013	Helper plasmid for conjugal transfer of pGP704 L (Km^r)	21
pBK-miniTn*7*-ΩSm1	pUC19-based delivery plasmid for miniTn*7*-ΩSm1 (Sm^r, Ap^r)	42
pUX-BF13	R6K replicon-based helper plasmid, providing the Tn*7* transposase proteins (Ap^r, mob$^+$)	4
pGP704mutY::tet	*mutY*::*tet*-sequence-containing SacI-KpnI fragment from pB1scrMutY-Tet cloned into pGP704L	67
pKSmutM	pBluescript KS(+) containing the PCR-amplified *mutM* gene region inserted in EcoRV-opened vector plasmid	This study
pKSΔmutM::km	*mutM* in pKSmutM is interrupted with the Km^r gene from pUTmini-Tn*5* Km2 by replacing PaeI- and Van9II-generated fragment from *mutM* by Km^r gene	This study
pGP704ΔmutM::km	pGP704 L with Acc65I-XbaI fragment of Δ*mutM*::*km* from pKSΔmutM::km in vector plasmid opened with the same restrictases	This study
pKSmutT	pBluescript KS(+) containing the PCR-amplified *mutT* gene region inserted into EcoRV-opened vector plasmid	This study
pKSΔmutT::km	*mutT* in pKSmutT is interrupted with Km^r gene from pUTmini-Tn*5* Km2 by replacing Bpu1102I-Van91I-generated fragment in *mutT* with Km^r gene	This study
pGP704ΔmutT::km	pGP704 L with Acc65I-XbaI fragment of Δ*mutT*::*km* from pKSΔmutT::km in vector plasmid opened with the same restrictases	This study
pKSdps	pBluescript KS(+) containing the PCR-amplified *dps* gene region inserted into EcoRV-opened vector plasmid	This study
pKSΔdps::km	*dps* in pKSdps is interrupted with Km^r gene from pUTmini-Tn*5* Km2 by replacing Eco130I-EcoRV-generated fragment in *dps* with Km^r gene	This study
pGP704Δdps::km	pGP704 L with Acc65I-XbaI fragment of Δ*dps*::*km* from pKSΔdps::km in vector plasmid opened with the same restrictases	This study
pUCNotrulAB	pUC18Not containing *rulAB* genes	74
pBK-miniTn*7*-ΩSm1rulAB	*rulAB* genes from pUC18NotrulAB in NotI site of pBK-miniTn*7*-ΩSmI	This study
pKTpheA56+A	Test system for detection of Phe$^+$ revertants occurring due to 1-bp deletions	76
pKTpheA22TAG	Test system for detection of Phe$^+$ revertants occurring due to base substitutions	75
pKT240	Medium-copy broad-host-range cloning vector (Ap^r Km^r)	3

codon of the *mutT* gene, and mutTalum (5′-TTGCAGAAGTGTTGTGGCAG C-3′), complementary to nucleotide sequence +79 to +59 downstream of the TGA stop codon of the *mutT* gene, were used for the amplification of the 942-bp *mutT* gene. The amplified 1,005-bp DNA fragment containing the *mutT* gene was inserted into the EcoRV-cleaved vector plasmid pBluescript KS(+) to obtain plasmid pKSmutT. The Km^r gene was amplified by PCR from plasmid pUTmini-Tn*5* Km2 by using the primer KmSac. The Ecl136II-cleaved DNA fragment containing the Km^r gene was used to replace the Bpu1102I- and Van91I-generated 450-bp fragment in the *mutT* gene in plasmid pKSmutT. The Bpu1102I ends were blunt ended before the ligation. The XbaI- and Acc65I-generated DNA fragment containing the Δ*mutT*::*Km* sequence from plasmid pKSΔmutT::Km was inserted into plasmid pGP704 L, cleaved with the Acc65I and XbaI enzymes. The resulting plasmid, pGP704ΔmutT::Km, was selected in *E. coli* strain CC118 λpir. The interrupted *mutT* gene containing the internal deletion was inserted into the chromosome of *P. putida* PaW85 by homologous recombination. The PaW85 Δ*mutT*::*km* knockout mutant PaWMutT was verified by PCR analysis using primers mutTlookus1 (5′-CCTACATCGAGACCATTTATCAG-3′), complementary to the nucleotide sequence from −73 to −51 upstream of the ATG initiator codon of the *mutT* gene, and KmOc (5′-TCGAGCAAGACGTTTCC C-3′), complementary to the nucleotide sequence 34 to 16 downstream of the ATG initiator codon of the Km^r gene.

In order to obtain independently isolated clones of *P. putida* PaW85 *mutY* knockouts, the previously constructed plasmid, pGP704mutY::tet (67), was used to carry out homologous recombination between the wild-type *mutY* allele and the interrupted one. The strain PaWMutY containing the disrupted *mutY* gene (*mutY*::*tet*) was verified by PCR analysis. In addition, the mutator phenotype of the clones containing the interrupted *mutY* sequence but lacking the original sequence was examined by measuring the spontaneous frequency of mutation to rifampin resistance.

Construction of *P. putida* strains carrying the DNA polymerase PolV homologue-encoding *rulAB* genes in chromosome. The plasmid pUCNotrulAB (74) carrying the *rulAB* operon, originated from the catabolic TOL plasmid pWW0

(29, 77), was used to subclone these genes as a NotI-cleaved DNA fragment into the NotI-cleaved mini-Tn*7* transposon delivery plasmid pBK-miniTn7-ΩSm1 (42). The plasmid pBK-miniTn7-ΩSm1rulAB, which is not able to replicate in *Pseudomonas* spp., was selected in *E. coli* strain DH5α and was further used in mobilization by four-parent conjugation into various *P. putida* strains. Plasmid pBK-miniTn7-ΩSm1rulAB carrying the *rulAB* genes was introduced into *P. putida* PaW85 by four-parent mating. More precisely, the following strains were used in four-parental mating: *E. coli* strain DH5α containing the delivery plasmid pBK-miniTn7-ΩSm1rulAB; *E. coli* CC118 λpir carrying the helper plasmid pUX-BF13 (4) for the transposition event to occur, as it contains the Tn*7* transposase genes; *E. coli* HB101 carrying the plasmid pRK2013 (the helper plasmid for other plasmids' mobilization); and *P. putida* strain PaW85. Consequently, integration of the *rulAB* genes in the composition of mini-Tn*7* into the specific *att*Tn*7* site located downstream of the *glmS* gene in *P. putida* chromosome was verified by PCR analysis with primers Tn7GlmS and Tn7R (43). The *P. putida* strain carrying the *rulAB* genes in its chromosome was designated as PaWRulAB. The expression of the *rulAB* genes in the *P. putida* chromosome was verified by using a UV irradiation survival assay described previously (74). In order to obtain *P. putida* GO repair-deficient strains carrying the *rulAB* genes in their chromosomes (strains PaWMutYRulAB, PaWMutMRulAB, and PaWMutTRulAB), PaWRulAB was used as a recipient for the replacement of original *mut* sequences with the interrupted ones by homologous recombination, as described above.

Construction of the *P. putida* PaW85 *dps* knockout strain. The *dps* (PP1210) gene sequence of *P. putida* KT2440 was obtained from the Institute for Genomic Research website (http://www.tigr.org). The 471-bp *dps* gene was amplified by PCR from the genomic DNA of *P. putida* strain PaW85. The primers dpsylem (5′-TTG AGTGCCGCGTGCCTT-3′), complementary to the nucleotide sequence −641 to −623 upstream of the ATG initiator codon of the *dps* gene, and dpsalum (5′-AG GTGGCCTATGAAGCGCTGA-3′), complementary to the nucleotide sequence +183 to +164 downstream of the TAA stop codon of the *dps* gene, were used for amplification of the *dps* gene. The amplified 1,350-bp DNA fragment containing the *dps* gene was inserted into the EcoRV-cleaved vector plasmid pBluescript KS(+) to obtain plasmid pKSdps. The Km^r gene was amplified by PCR from plasmid pUTmini-Tn*5* Km2 by using the primer KmSac. The EcI136II-cleaved DNA fragment containing the Km^r gene was used to replace the Eco130I- and EcoRV-generated 200-bp fragment in the *dps* gene in plasmid pKSdps. The ends of the Eco130I were blunt ended before the ligation reaction. The XbaI- and Acc65I-generated DNA fragment containing the *Δdps*::*Km* sequence from plasmid pKSΔdps::Km was inserted into plasmid pGP704 L, cleaved with the same enzymes. The resulting plasmid, pGP704Δdps::Km, was selected in *E. coli* strain CC118 λpir. The interrupted *dps* gene containing the internal deletion was inserted into the chromosome of *P. putida* PaW85 by homologous recombination. Plasmid pGP704Δdps::km, not able to replicate in hosts other than *E. coli* CC118 λpir, was conjugatively transferred into *P. putida* PaW85 by using helper plasmid pRK2013. The PaW85 *Δdps*::*km* knockout mutant PaWDps was verified by PCR analysis using primers dpslookus (5′-CGGTTGCCGGCATCTTGTG-3′), complementary to the nucleotide sequence −667 to −649 upstream of the ATG initiator codon of the *dps* gene, and KmOc (5′-TCGAGCAAGACGTTTCCC-3′), complementary to the nucleotide sequence 34 to 16 downstream of the ATG initiator codon of Km^r gene.

Isolation and analysis of Phe$^+$ revertants and Rif^r mutants. Assay systems used to measure different types of point mutations in starving *P. putida* were based on the activation of phenol monooxygenase gene *pheA*, enabling bacteria to utilize phenol as the growth substrate and to form colonies on selective plates. The reporter gene *pheA* was altered in RSF1010-derived tester plasmids either by +1 frameshift mutation or by introducing a TAG translational stop codon into the *pheA* gene (75). Conditions for the isolation of phenol-degrading Phe$^+$ revertants were the same as those described in our previous study (66). Carbenicillin at a concentration of 1,500 μg/ml was used in selective plates to maintain selectin for plasmid. Several independently isolated clones of the same gene knockouts were used in mutagenesis studies to control reproducibility of the results. We have previously shown (40, 75) that Phe$^+$ colonies appearing on phenol-minimal plates on day 2 contained mutations that occurred before the plating in a growing culture, whereas colonies that emerged on selective plates on day 3 and later contained mutations that occurred after the cells were plated. The latter were called stationary-phase mutations. About 5×10^8 cells of the *P. putida* wild-type strain and its derivatives were plated onto phenol-minimal plates from independent cultures that were gown overnight in liquid M9 medium containing glucose and CAA. When larger amounts of cells of the tester strains were plated onto selective plates, they were plated with approximately 5×10^8 scavenger cells (*P. putida* PaW85 carrying pKT240 [3]). In order to test whether bacteria would die during starvation on the selective medium, we measured the number of CFU for at least five independent starving cultures of the particular strain studied. The viability of bacteria was determined on the same plates that were used for the isolation of Phe$^+$ revertants. No differences were found between the viability of wild-type strain PaW85 and that of its derivatives during incubation of bacteria under starvation conditions.

The frequency of spontaneous mutations in growing cells was determined in at least four separate experiments, in each of 20 independent cultures. Independent cultures of *P. putida* tester strains were generated by growing cells to late logarithmic growth phase in M9 medium containing glucose and CAA, diluting this culture by 10^5 into fresh glucose and CAA-containing M9 medium, and dispensing 2-ml aliquots into test tubes and allowing cells to reach saturation by growing cells for 18 to 20 h. Cells sampled from the same cultures were used for determination of the frequency of Phe$^+$ revertants and Rif^r mutants. Phe$^+$ revertants appearing on phenol-minimal plates were counted on day 2 after plating. To determine the frequency of Rif^r mutants, colonies were counted on 100-μg/ml-rifampin-supplemented plates incubated for 24 h.

The frequency of spontaneous mutations in independently growing cultures was calculated per 1×10^9 plated cells by using the Lea-Coulson method of the median (44, 63). Data were analyzed by a software program for statistical analyses (Statgraphics Centurion XV; Statpoint Inc.). *P* values of the medians for Rif^r or Phe$^+$ mutations were calculated by using the Mann-Whitney (Wilcoxon) W test (71). Differences between average accumulation rates of Phe$^+$ mutants were analyzed using the Student *t* test. The chi-square test of independence was used for comparing DNA sequencing results.

For Phe$^+$ revertants, an approximately 350-bp DNA region covering the area of the *pheA* gene containing potential reversion mutations was analyzed by DNA sequencing, as described previously (75). The DNA sequencing reactions were analyzed with an ABI Prism 377 DNA sequencer (Perkin-Elmer).

Oxidative-stress assay. The oxidative stress assay was performed as described in reference 56, with some modifications. The overnight stationary-phase cultures of wild-type *P. putida* strain PaW85 and its *dps*-defective derivative were incubated in 30 ml of LB broth at 30°C with aeration in the presence or absence of hydrogen peroxide (Sigma) at a concentration of 200 mM. After the addition of hydrogen peroxide, viable cell counts were determined as CFU, by serial dilution of cells eliminated periodically from cultures and plated to the LB agar plates. All assays were done at least four times.

RESULTS AND DISCUSSION

Mutation frequency in growing cells of *P. putida* strains lacking different GO repair enzymes. The GO system has been studied mainly in *E. coli*, and it is known to involve at least three enzymes, MutY and MutM glycosylases and MutT pyrophosphohydrolase (52). The absence of MutY or MutT causes a strong mutator phenotype in *E. coli*, whereas the lack of MutM has a milder effect on mutation frequency (17, 45, 59, 81). Mutators isolated from natural populations of *Pseudomonas* species have been found to be defective in either enzymes of DNA mismatch repair or MutY, but no mutators were identified with inactivation of the *mutT* or *mutM* gene (20, 60, 61). This raised the question of how essential MutT and MutM are in the avoidance of mutations in pseudomonads. We have previously shown that the lack of MutY leads to the mutator phenotype in *P. putida* (67). To further elucidate the role of the GO repair system in the avoidance of mutations in this organism, we constructed *P. putida* strains PaWMutM and PaWMutT, which were defective in MutM or MutT, respectively. MutT, first characterized in *E. coli* (9, 45), belongs to the Nudix family of hydrolases. Enzymes containing the Nudix box invariably catalyze the hydrolysis of nucleoside diphosphates linked to some other moiety, X (7, 50). According to annotation of the *P. putida* genome at the Institute for Genomic Research website (http://www.tigr.org), this organism contains several open reading frames encoding putative Nudix family (known also as MutT family) proteins. Based on DNA sequence data, PP1062 and PP1348 are the closest homologues to *E. coli mutT* (they share 41% identity at the deduced amino

TABLE 2. Effect of inactivation of GO repair enzymes on frequency of mutations in growing cells of *P. putida*[a]

Strain genotype	Median frequency for Rif^r mutation	Phenotypic effect[b]	Median frequency for Phe$^+$ mutation	Phenotypic effect[b]
WT	3.8	1	1.5	1
mutY	345	91	120	80
Δ*mutM*	8.4	2.2	2	1.3
Δ*mutT*	11	2.9	2	1.3

[a] The median value for frequency of mutation per 1×10^9 cells is shown. This was calculated using the Lea-Coulson method of the median (44, 63). The frequency of mutation was determined for at least 60 independent cultures. Differences between frequencies of Rif^r mutations of PaWMutY, PaWMutM, and PaWMutT relative to that of the wild-type strain and Phe$^+$ mutations of PaWMutY relative to that of the wild-type strain were statistically significant (*P* value less than 0.05) at the 95% confidence level, based on the Mann-Whitney test (71).

[b] The phenotypic effect is given as a ratio calculated by dividing the mutation frequency of a particular GO repair-deficient strain by the mutation frequency of wild-type strain PaW85.

acid level with *E. coli mutT*). Among the 10 different genes encoding putative Nudix hydrolases, PP1348 is the closest homologue to the *P. aeruginosa mutT* gene PA4400 (they share 72% identity at the deduced amino acid sequence level), which has been demonstrated to complement *E. coli mutT* deficiency (61). Hence, PP1348 was chosen as the target to knock out MutT activity in *P. putida*. Construction of GO repair-deficient strains is described in detail in Materials and Methods.

We examined the frequency of spontaneous mutations of growing cells in the *P. putida* wild-type strain and its GO repair-defective derivatives, using two different assay systems. The chromosomal Rif^r system enabled the detection of Rif^r colonies occurring due to mutations in the *rpoB* gene. The other test system measured base substitutions by eliminating the TAG stop codon in the phenol monooxygenase (*pheA*) gene present in the RSF1010-derived plasmid (75). Point mutations that restored the functional *pheA* sequence enabled bacteria to utilize phenol as a growth substrate (we counted Phe$^+$ colonies appearing on day 2 after plating onto phenol-minimal plates). The results presented in Table 2 show that the median values of frequency of spontaneous Rif^r mutations and Phe$^+$ mutations were increased ~90-fold and ~80-fold, respectively, in PaWMutY compared to that of the wild-type strain. At the same time, the absence of MutM or MutT elevated the frequency of Rif^r mutations by only two- to threefold. No significant effect of the lack of MutM or MutT on the frequency of mutations was detected with the test system which measured the appearance of Phe$^+$ revertants. Thus, our results clearly demonstrated that the lack of MutY activity and not the knockout of MutM or MutT caused the mutator phenotype in growing cells of *P. putida*.

Roles of MutT and MutM in stationary-phase mutagenesis of *P. putida*. We have previously shown that the absence of MutY had a strongly increasing effect on the frequency of mutations in starved populations of *P. putida* (67). To study the effect of various GO repair enzymes on the avoidance of stationary-phase mutations in *P. putida*, we used different assay systems that enabled us to monitor base substitutions and frameshift mutations separately (75). Phe$^+$ revertants accumulating on phenol-minimal plates on day 3 and later were counted on each day during 2 weeks of starvation. We found that the frequency of accumulation of 1-bp deletion mutants was not affected by the absence of GO repair enzymes (data not shown). At the same time, the deficiency of either MutY or MutT greatly elevated the occurrence of base substitution mutations, resulting in about 100-fold and 75-fold higher frequencies of accumulation of Phe$^+$ revertants, respectively, than that

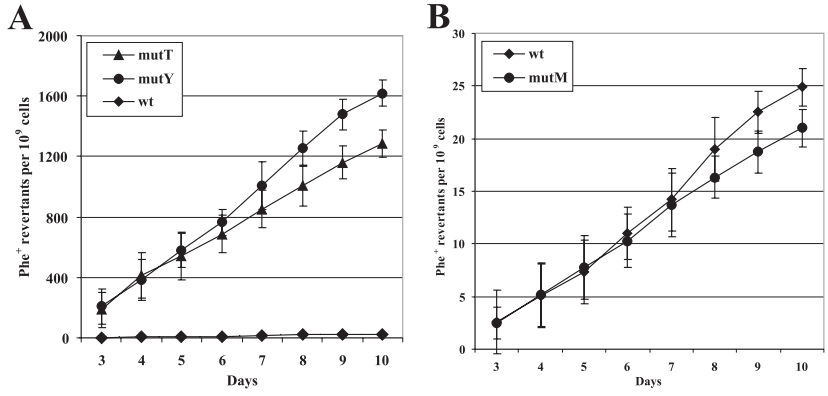

FIG. 1. Accumulation of Phe$^+$ revertants on phenol-minimal plates in *P. putida* wild-type strain PaW85 (wt) and in its GO repair-defective derivatives PaWMutY (mutY), PaWMutT (mutT), and PaWMutM (mutM). (A) Accumulation of stationary-phase mutations in strains PaW85, PaWMutY, and PaWMutT. (B) Accumulation of stationary-phase mutations in strains PaW85 and PaWMutM. About 2×10^7 cells of tester strain PaWMutY or PaWMutT carrying the plasmid pKTpheA22TAG with 5×10^8 scavenger cells (PaW85 cells carrying pKT240) or 5×10^8 cells of tester strain PaW85 or PaWMutM carrying the plasmid pKTpheA22TAG were plated from independent liquid M9 medium cultures grown overnight onto phenol-minimal plates. Data for at least five parallel experiments are presented. In all cases, means ± standard deviations (error bars) for at least 10 plates calculated per 1×10^9 cells are shown.

TABLE 3. Reversion of nonsense mutation (TAG) in Phe$^+$ mutants accumulating in *P. putida* wild-type strain and its GO repair-defective derivatives

Target[a]	DNA change	Occurrences (%)				Occurrences with *rulAB* genes inserted into chromosome (%)			
		Wild type	*mutM*	*mutT*	*mutY*	Wild type	*mutM*	*mutT*	*mutY*
TAG	T → C	164 (77)	103 (66)	14 (8.3)	24 (12)	**115 (60)**[c]	59 (63)	7 (5)	23 (12)
	T → G	19 (9)	11 (7)	34 (20)	5 (2)	11 (6)	4 (4.3)	36 (25)	5 (2.5)
	T → A	1 (0.5)	0	0	0	0	1 (1.1)	0	0
	G → T	12 (5.6)	**24 (15.4)**[b]	1 (0.6)	176 (85)	6 (3)	16 (17.2)	0	168 (84)
	A → C	0	3 (2)	118 (70)	0	**4 (2)**[c]	**7 (8)**[c]	102 (70)	1 (0.5)
	A → G	13 (6)	10 (6.4)	2 (1.1)	3 (1)	**51 (27)**[c]	3 (3.2)	0	1 (0.5)
	A → T	4 (1.9)	5 (3.2)	0	0	4 (2)	3 (3.2)	0	1 (0.5)

[a] Phe$^+$ mutant colonies used for identification of stationary-phase mutations were picked up on days 3 to 15. Approximately 15 mutants were analyzed per each day. We did not notice remarkable changes in the spectrum of mutations in revertants derived from the earlier or the later period of starvation.
[b] Bold type denotes statistically significant differences ($P < 0.05$) between the *mutM* and wild-type strains (marked also in bold).
[c] Bold type denotes statistically significant differences ($P < 0.05$) in the presence or absence of *rulAB* genes (marked also in bold).

of wild-type bacteria (Fig. 1A). However, bacteria with the *mutM*-defective allele did not reveal statistically significant changes in frequency of stationary-phase mutations compared to that of the wild-type strain (Fig. 1B). These data demonstrated that both MutY and MutT are important in decreasing the rate of mutation in starving *P. putida*.

Analysis of the Phe$^+$ revertants which appeared due to base substitutions revealed that the spectrum of mutations identified in the wild-type strain was distinct from the spectrum of changes characterized with different GO repair-defective strains (Table 3). Whereas the T-to-C transition was the most prominent change in wild-type bacteria (77% among all changes identified), the revertants which were collected from populations of MutY-defective bacteria contained predominantly G-to-T transversions (85%). Revertants isolated from MutT-defective bacteria contained mostly two types of transversions, A-to-C (70%) or T-to-G (20%). These data are consistent with known functions of MutY and MutT in *E. coli* (54).

MutT hydrolyzes 8-oxodGTP to prevent its incorporation into DNA (45). Our results demonstrated that *P. putida* carrying the *mutT* (PP1348) null allele expressed a strong mutator phenotype in carbon-starved bacteria but affected only slightly the mutation frequency in growing bacteria. Annotation of the *P. putida* genome (www.tigr.org) indicates the presence of 10 putative Nudix family proteins in this organism. Some Nudix family members, such as MutT, are known to have the ability to degrade potentially mutagenic, oxidized nucleotides, while others control the levels of metabolic intermediates and signaling molecules (50). The presence of multiple genes for Nudix family proteins is common in bacteria. For example, *E. coli* has 13 Nudix hydrolases (including MutT), 11 of which are characterized by their enzymatic activities (50, 79). Inactivation of the *mutT* gene in *E. coli* leads to an approximately 1,000-fold increase in spontaneous mutation frequency due to increased A · T-to-C · G transversions (81). Studies over the past 10 years have revealed that at least three other proteins besides MutT could eliminate 8-oxodGTP from the nucleotide pool in growing *E. coli* cells (34, 37, 41). However, compared to MutT, the antimutator effect of these proteins is considerably weaker (about two- to threefold effects). Orf135 (NudG) is a Nudix family protein which hydrolyzes 2-hydroxy-dATP and 8-oxoG (39). The lack of Orf135 enzyme resulted in a mild mutator phenotype, and overexpression of Orf135 protein reduced mutation frequency in *E. coli* (37). Overexpression of Orf17 (NtpA, NudB) protein, another Nudix family enzyme, decreased mutation frequency in MutT-defective *E. coli* (34). Additionally, GTP cyclohydrolase II, which is not related to Nudix family proteins, may also serve as a backup enzyme for the MutT protein, since it is able to hydrolyze 8-oxoG and its absence increases mutation frequency in MutT-defective *E. coli* (41). The possible antimutator function of these proteins in starving bacteria has not been studied. The biological function and substrate specificity of *P. putida* Nudix family proteins are entirely unexplored. Based on results of the current study, we hypothesize that the role of some of the other nine putative *P. putida* Nudix family proteins is to back up the PP1348-encoded MutT function in a growth phase-dependent manner. It is possible that any of these proteins not expressed in starving bacteria but active in growing bacteria may suppress the lack of PP1348-encoded activity in growing *P. putida*.

A search of the www.tigr.org website revealed that the *P. aeruginosa* PAO1 genome encodes nine putative Nudix family proteins. Drawing parallels with the results of our study demonstrating that the absence of the PP1348-encoded MutT homologue had only a mild effect on mutation frequency in growing bacteria, we propose that similarly to *P. putida*, some other Nudix protein(s) could serve as a backup for MutT in *P. aeruginosa*. This might be a reason why no *mutT*-defective variants were identified among *P. aeruginosa* clinical isolates expressing a strong mutator phenotype (see, e.g., references 60 and 61).

Although the absence of MutM did not elevate the mutation frequency in starving *P. putida*, the proportion of G-to-T transversions increased significantly in this strain (15.4% were identified in PaWMutM versus 5.6% detected in the wild-type strain [$P = 0.0018$]) (Table 3). This hints that MutM actually is functional in starving *P. putida*. Compared to the other GO repair system enzymes MutY and MutT, a deficiency in MutM also had a much smaller effect on stationary-phase mutations in other bacterial species including the best-studied microorganism, *E. coli* (15). *E. coli* has additional enzymes to remove 8-oxoG from mispairs. Hazra et al. (31) found that the Nei protein (endonuclease VIII) could excise 8-oxoG from 8-oxoG/A and 8-oxoG/G mispair-containing oligonucleotides. Triple mutants lacking MutY, MutM, and Nei showed an increase in G · C-to-T · A transversions, which indicated that *nei* serves as a backup to remove 8-oxoG (11). Additionally, the *E.*

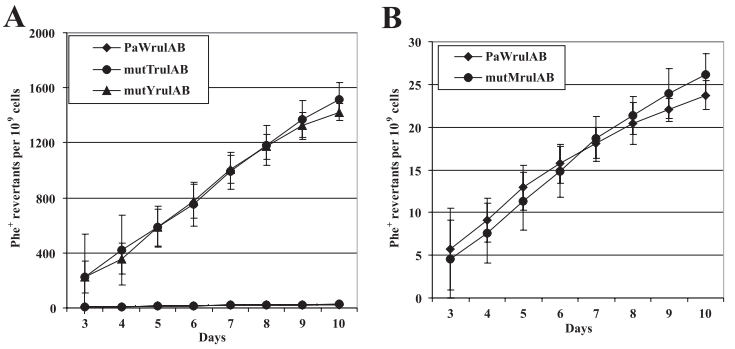

FIG. 2. Study of the effect of *rulAB* genes on the accumulation of Phe^+ revertants on phenol-minimal plates in *P. putida* wild-type strain PaW85 (PaWrulAB) and in its GO repair-defective derivatives PaWMutY (mutYrulAB), PaWMutT (mutTrulAB), and PaWMutM (mutMrulAB). (A) Accumulation of stationary-phase mutations in strains PaWRulAB, PaWMutYRulAB, and PaWMutTRulAB. (B) Accumulation of stationary-phase mutations in strains PaW85RulAB and PaWMutMRulAB. Data for at least five parallel experiments are presented. In all cases, means ± standard deviations (error bars) for at least 10 plates calculated per 1×10^9 cells are shown. Differences between average accumulation rates of Phe^+ mutations per day per 1×10^9 cells measured in the presence of the *rulAB* genes during a 3- to 10-day starvation period were not statistically significant (except for strain PaWMutYRulAB) compared to those measured in counterpart strains without the *rulAB* genes ($P = 0.17$ for PaW85; $P = 0.27$ for PaWMutM; $P = 0.31$ for PaWMutT; and $P < 0.001$ for PaWMutY) at the 95% confidence level, based on the Student *t* test.

coli Nth protein (endonuclease III) has an 8-oxoG DNA glycosylase/AP lyase activity which removes 8-oxoG preferentially from 8-oxoG/G mispairs (48). Mutants defective in Nei exhibited no mutator phenotype, and mutants defective in Nth showed a weak mutator phenotype, while double mutants lacking both Nei and Nth exhibited a strong mutator phenotype (35, 65). Annotation of the *P. putida* genome did not indicate the presence of putative endonuclease VIII (Nei) homologues in this organism but did reveal that *P. putida* has an Nth homologue. The deduced amino acid sequence of PP1092 showed 67.8% identity with the sequence of *E. coli* endonuclease III (Nth protein). Thus, one may hypothesize that the *P. putida* Nth homologue may suppress a mutator phenotype of *P. putida* lacking the functional *mutM* gene.

The DNA polymerase PolV homologue RulAB influences the spectrum of stationary-phase mutations. It was reported that stationary-phase populations of MutY-defective *E. coli* had, in addition to G · C-to-T · A transversions, an enhanced rate of G · C-to-C · G transversions, and evidence was presented that MutY protein possesses a DNA glycosylase activity to remove unmodified G from an 8-oxoG · G mispair (83). The study by Timms et al. (76) revealed that the pathway which generates G · C-to-C · G transversions in starved *E. coli* requires the *umuDC* genes encoding DNA polymerase PolV. PolV is involved in the appearance of mutants able to utilize novel growth substrates and mutants with enhanced abilities to scavenge amino acids released from dead cells in starving populations of *E. coli* (8, 16, 49, 82). Similar results have been obtained with *P. putida*. We have recently shown that the PolV homologue encoded by TOL plasmid pWW0 significantly enhances the fitness of bacteria under conditions of long-term starvation (74). These results indicated that PolV genes *rulAB* from the TOL plasmid increase the probability of accumulation of beneficial mutations in *P. putida* cells, allowing genetic adaptation of bacterial populations under conditions of environmental stress. Here, in order to study the possible relationship between RulAB-dependent mutagenesis and oxidative damage of DNA in starving *P. putida*, we looked at the effect of the *rulAB* genes on the frequency of mutations and spectra of base substitution mutations in a *P. putida* wild-type strain and in its GO repair-defective derivatives. The frequency of accumulation of Phe^+ revertants in the *P. putida* wild-type strain and its MutY-, MutM- or MutT-defective derivative in the presence of the *rulAB* genes is shown in Fig. 2. The presence of the *rulAB* genes did not significantly affect the accumulation rate of Phe^+ revertants compared to that measured in strains lacking these genes, except for MutY-defective bacteria, where the accumulation rate of the Phe^+ revertants was slightly reduced in the *rulAB*-proficient strain PaWMutYRulAB compared to that in strain PaWMutY. However, a comparison of results of the DNA sequence analysis of Phe^+ mutants accumulated in the presence or absence of the *rulAB* genes in the bacterial chromosome revealed differences between the spectra of base substitutions identified in the wild-type strain and those in the strain lacking MutM (Table 3). These data demonstrated that *rulAB*-encoded PolV contributes to the occurrence of base substitution mutations in stationary-phase populations of *P. putida*. At the same time, and differing from our expectations, this DNA polymerase did not increase the proportion of G-to-C substitutions. Instead, the presence of the *rulAB* genes in the bacterial chromosome enhanced the replacement of nucleotide A with nucleotide G or C. In GO repair-proficient bacteria, the proportion of A-to-G transitions was increased from 6% in the PaW85 strain lacking *rulAB* genes to 27% in the PaWRulAB strain carrying *rulAB* genes ($P < 0.001$). There was also an increase in A-to-C transversions in the following strains: the proportion of A-to-C changes was 2% in strain PaWRulAB, whereas A-to-C substitutions were not detected among 213 mutants picked up from starved populations of strain PaW85 ($P = 0.03$). In the case of

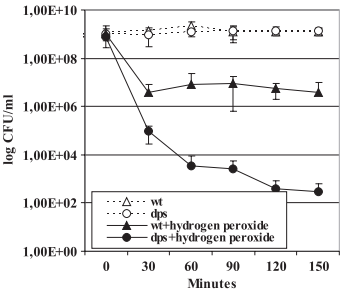

FIG. 3. Effect of the absence of Dps on survival of bacteria in the presence of 200 mM H_2O_2. Overnight cultures of wild-type (wt) or Dps-defective (dps) cells were treated with H_2O_2. After the addition of hydrogen peroxide, viable cell counts were determined as CFU by serial dilution of cells eliminated periodically from cultures and plated to the LB agar plates. Open symbols designate nontreated cells. All assays were done at least four times.

a MutM-defective background, the presence of the *rulAB* genes increased the proportion of A-to-C transversions from 2%, identified in bacteria in the absence of RulAB (strain PaWMutM), to 8% in RulAB-proficient cells (strain PaWMut MRulAB) ($P = 0.029$). Notably, the frequency of A-to-C substitutions was not affected by the presence of *rulAB* genes in MutT-defective bacteria (Table 3). This indicates that this type of transversion was not caused by misincorporation of 8-oxoG opposite template A.

So far, in contrast to 8-oxoG, 2-hydroxyadenine (2-OH-A), an oxidized form of adenine, has received less attention because its steady-state levels in cellular DNA are lower than those of 8-oxoG (25, 38). However, 2-OH-A is as mutagenic as 8-oxoG is (36). Most recently, it was demonstrated that replication fork block is the likely outcome of a replicative DNA polymerase encountering a template 2-OH-A, and its bypass by translesion synthesis polymerases is mutagenic (5). Bypass of 2-OH-A by two Y family polymerases, archaeal polymerase Dpo4 and human polymerase η, was associated with A-to-G, A-to-C, and A-to-T base substitutions (5). Since PolV also belongs to a Y family of DNA polymerases and plays a crucial role in carrying out translesion synthesis past damaged bases in DNA, one may speculate that the effects of RulAB observed in our studies are due to the presence of oxidatively damaged adenine in DNA. Interestingly, the proportion of A-to-G transitions also increased in starved cells of *P. putida* lacking DNA MMR (66). Notably, Barone et al. (5) have suggested that MMR would prevent the accumulation of 2-OH-A in DNA and thereby the accumulation of A-to-G and A-to-T base substitutions. Whether this is also true in our case needs further studies.

Paradoxically, the proportion of A-to-C transversions also increased in MutM-defective bacteria. According to the known substrate specificity of MutM, this enzyme, in addition to 8-oxoG, repairs a number of alternative substrates (e.g., see references 27 and 30). However, to our knowledge, no 2-OH-A glycosylase activity has been reported for MutM. Thus, the mechanism of enhancement of A-to-C transversions in MutM-defective bacteria in the presence of RulAB remains obscure.

Dps does not affect stationary-phase mutations in *P. putida*. Dps, a stationary-phase-specific DNA-binding protein, is present in a wide variety of organisms. It has been demonstrated that Dps protects cells against multiple stresses during the stationary phase (56). Among these various functions, Dps protects DNA against oxidative damage by serving as an alternative target for reactive oxygen species (26, 46, 56, 78). So far, the effect of Dps on mutagenic processes has been investigated only in the presence of exogenously added hydrogen peroxide and by overexpressing this enzyme in GO repair-deficient *E.*

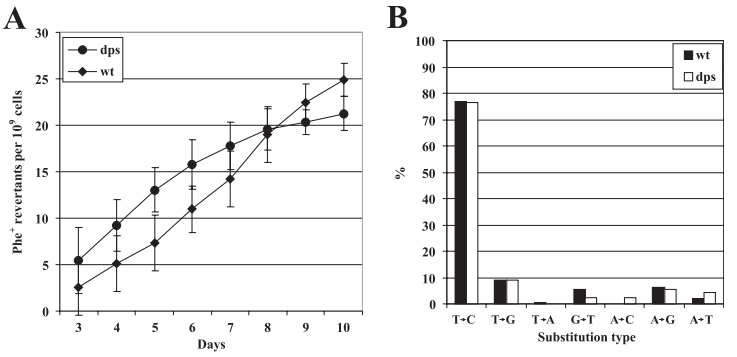

FIG. 4. Study of the effect of Dps on the occurrence of stationary-phase mutations in *P. putida*. (A) Accumulation of Phe^+ revertants on phenol-minimal plates of *P. putida* wild-type strain (wt) and its Dps-defective derivative PaWDps (dps). Data for at least five parallel experiments are presented. In both cases, means ± standard deviations (error bars) for at least 10 plates calculated per 1×10^9 cells are shown. Differences between average accumulation rates of Phe^+ mutations per day per 1×10^9 cells measured in these strains during 3- to 10-day starvation periods are not statistically significant (P value of 0.77) at the 95% confidence level, based on the Student t test. (B) The spectrum of Phe^+ mutations occurring in *P. putida* wild-type strain and its Dps-defective derivative PaWDps (dps). Minor differences in the spectra of base substitutions seen in this figure are not statistically significant ($P > 0.05$).

coli (46). In that particular study, the lack of Dps resulted in an approximately 10-fold increase in G · C-to-T · A transversions (46). Whether Dps prevents mutations under physiological conditions was not investigated in that report. Hence, we decided to examine whether Dps could have an effect on the frequency of stationary-phase mutations in *P. putida*. For that purpose, we constructed a Dps-defective mutant of *P. putida*. First, in order to determine whether Dps is involved in the protection of *P. putida* against oxidative damage, we performed a hydrogen peroxide sensitivity test of the *P. putida* wild-type strain and its Dps-defective derivative strain PaWDps. We found that strain PaWDps was more sensitive to hydrogen peroxide stress than the wild type (Fig. 3). The viability of PaWDps treated with 200 mM H_2O_2 was reduced about 10^4-fold after 30 min of treatment compared to about 300-fold reduction in viability of wild-type cells. Later, wild-type bacteria adapted to this stress as they maintained viability at 4×10^6 CFU/ml for 150 min. Adaptation to hydrogen peroxide stress also took place in cultures of bacteria lacking Dps: viable cell titers of Dps-defective bacteria were reduced by more than 10^6-fold for 150 min (we detected 3×10^2 CFU/ml), but this decline was much slower than the rapid death that occurred during the first 30 min after the addition of hydrogen peroxide to the growth medium of bacteria. These data indicate that Dps is involved in the protection of *P. putida* cells against sudden exposure to H_2O_2.

Next, we compared the frequencies of accumulation of Phe^+ revertants from starving populations of the *P. putida* wild-type strain with those of its Dps-defective derivative strain PaWDps (Fig. 4A). Although the curves of accumulation of the mutants seem slightly different, these differences appeared not to be statistically significant. Additionally, the spectrum of base substitutions occurring in starving populations of Dps-defective bacteria was similar to that of the wild-type bacteria (Fig. 4B). Here we can state that the introduction of the *dps* null allele into *mutY*-defective bacteria also did not elevate the rate of accumulation of Phe^+ revertants (data not shown). Thus, our results imply that Dps has no role in the avoidance of stationary-phase mutations, at least in *P. putida*. It is also possible that the protective role of Dps in genome integrity becomes essential only when bacteria are exposed to exogenous DNA-damaging agents.

To summarize our results, we conclude that the oxidative damage of DNA is an important source of stationary-phase mutagenesis in *P. putida*. The lack of activity of the GO repair system significantly increases the frequency of base substitutions in starved populations of *P. putida*. However, although Dps protects *P. putida* against oxidative stress, Dps did not counteract mutagenesis under starvation conditions of bacteria. Additionally, the presence of the Y family DNA polymerase PolV homologue elevated the frequency of A-to-C and A-to-G base substitutions, which might be a consequence of the presence of oxidized adenine on DNA. Hence, it would be interesting to study whether an enzyme(s) which may serve as a backup for MutM or any of numerous Nudix family proteins present in *P. putida* could be involved in avoidance of mutations caused by oxidative damage.

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REFERENCES

1. **Adams, M. H.** 1959. Bacteriophages. Interscience Publishers Inc., New York, NY.
2. **Almiron, M., A. J. Link, D. Furlong, and R. Kolter.** 1992. A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. Genes Dev. **6:**2646–2654.
3. **Bagdasarian, M. M., E. Amann, R. Lurz, B. Ruckert, and M. Bagdasarian.** 1983. Activity of the hybrid *trp-lac (tac)* promoter of *Escherichia coli* in *Pseudomonas putida*. Construction of broad-host-range, controlled-expression vectors. Gene **26:**273–282.
4. **Bao, Y., D. P. Lies, H. Fu, and G. P. Roberts.** 1991. An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. Gene **109:**167–168.
5. **Barone, F., S. D. McCulloch, P. Macpherson, G. Maga, M. Yamada, T. Nohmi, A. Minoprio, F. Mazzei, T. A. Kunkel, P. Karran, and M. Bignami.** 2007. Replication of 2-hydroxyadenine-containing DNA and recognition by human MutSalpha. DNA Repair **6:**355–366.
6. **Bayley, S. A., C. J. Duggleby, M. J. Worsey, P. A. Williams, K. G. Hardy, and P. Broda.** 1977. Two modes of loss of the Tol function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. **154:**203–204.
7. **Bessman, M. J., D. N. Frick, and S. F. O'Handley.** 1996. The MutT proteins or "Nudix" hydrolases, a family of versatile, widely distributed, "housecleaning" enzymes. J. Biol. Chem. **271**:25059–25062.
8. **Bhamre, S., B. B. Gadea, C. A. Koyama, S. J. White, and R. G. Fowler.** 2001. An aerobic *recA*-, umuC-dependent pathway of spontaneous base-pair substitution mutagenesis in *Escherichia coli*. Mutat. Res. **473:**229–247.
9. **Bhatnagar, S. K., and M. J. Bessman.** 1988. Studies on the mutator gene, mutT of *Escherichia coli*. Molecular cloning of the gene, purification of the gene product, and identification of a novel nucleoside triphosphatase. J. Biol. Chem. **263:**8953–8957.
10. **Bjelland, S., and E. Seeberg.** 2003. Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. Mutat. Res. **531:**37–80.
11. **Blaisdell, J. O., Z. Hatahet, and S. S. Wallace.** 1999. A novel role for *Escherichia coli* endonuclease VIII in prevention of spontaneous G→T transversions. J. Bacteriol. **181:**6396–6402.
12. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
13. **Bridges, B. A.** 1996. Mutation in resting cells: the role of endogenous DNA damage. Cancer Surv. **28:**155–167.
14. **Bridges, B. A.** 1993. Spontaneous mutation in stationary-phase *Escherichia coli* WP2 carrying various DNA repair alleles. Mutat. Res. **302:**173–176.
15. **Bridges, B. A., M. Sekiguchi, and T. Tajiri.** 1996. Effect of *mutY* and *mutM*/*fpg*-1 mutations on starvation-associated mutation in *Escherichia coli*: implications for the role of 7,8-dihydro-8-oxoguanine. Mol. Gen. Genet. **251:**352–357.
16. **Bull, H. J., M. J. Lombardo, and S. M. Rosenberg.** 2001. Stationary-phase mutation in the bacterial chromosome: recombination protein and DNA polymerase IV dependence. Proc. Natl. Acad. Sci. USA **98:**8334–8341.
17. **Cabrera, M., Y. Nghiem, and J. H. Miller.** 1988. *mutM*, a second mutator locus in *Escherichia coli* that generates G · C → T · A transversions. J. Bacteriol. **170:**5405–5407.
18. **Carter, P., H. Bedouelle, and G. Winter.** 1985. Improved oligonucleotide site-directed mutagenesis using M13 vectors. Nucleic Acids Res. **13:**4431–4443.
19. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
20. **de Weert, S., L. C. Dekkers, I. Kuiper, G. V. Bloemberg, and B. J. Lugtenberg.** 2004. Generation of enhanced competitive root-tip-colonizing *Pseudomonas* bacteria through accelerated evolution. J. Bacteriol. **186:**3153–3159.
21. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
22. **Foster, P. L.** 1993. Adaptive mutation: the uses of adversity. Annu. Rev. Microbiol. **47:**467–504.
23. **Foster, P. L.** 1999. Mechanisms of stationary phase mutation: a decade of adaptive mutation. Annu. Rev. Genet. **33:**57–88.
24. **Foster, P. L.** 2005. Stress responses and genetic variation in bacteria. Mutat. Res. **569:**3–11.
25. **Frelon, S., T. Douki, and J. Cadet.** 2002. Radical oxidation of the adenine

moiety of nucleoside and DNA: 2-hydroxy-2′-deoxyadenosine is a minor decomposition product. Free Radic. Res. **36:**499–508.
26. **Frenkiel-Krispin, D., S. Levin-Zaidman, E. Shimoni, S. G. Wolf, E. J. Wachtel, T. Arad, S. E. Finkel, R. Kolter, and A. Minsky.** 2001. Regulated phase transitions of bacterial chromatin: a non-enzymatic pathway for generic DNA protection. EMBO J. **20:**1184–1191.
27. **Fromme, J. C., and G. L. Verdine.** 2003. DNA lesion recognition by the bacterial repair enzyme MutM. J. Biol. Chem. **278:**51543–51548.
28. **Goodman, M. F.** 2002. Error-prone repair DNA polymerases in prokaryotes and eukaryotes. Annu. Rev. Biochem. **71:**17–50.
29. **Greated, A., L. Lambertsen, P. A. Williams, and C. M. Thomas.** 2002. Complete sequence of the IncP-9 TOL plasmid pWW0 from *Pseudomonas putida*. Environ. Microbiol. **4:**856–871.
30. **Hatahet, Z., Y. W. Kow, A. A. Purmal, R. P. Cunningham, and S. S. Wallace.** 1994. New substrates for old enzymes. 5-Hydroxy-2′-deoxycytidine and 5-hydroxy-2′-deoxyuridine are substrates for *Escherichia coli* endonuclease III and formamidopyrimidine DNA N-glycosylase, while 5-hydroxy-2′-deoxyuridine is a substrate for uracil DNA N-glycosylase. J. Biol. Chem. **269:**18814–18820.
31. **Hazra, T. K., T. Izumi, R. Venkataraman, Y. W. Kow, M. Dizdaroglu, and S. Mitra.** 2000. Characterization of a novel 8-oxoguanine-DNA glycosylase activity in *Escherichia coli* and identification of the enzyme as endonuclease VIII. J. Biol. Chem. **275:**27762–27767.
32. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172**:6557–6567.
33. **Hõrak, R., H. Ilves, P. Pruunsild, M. Kuljus, and M. Kivisaar.** 2004. The ColR-ColS two-component signal transduction system is involved in regulation of Tn*4652* transposition in *Pseudomonas putida* under starvation conditions. Mol. Microbiol. **54:**795–807.
34. **Hori, M., T. Asanuma, O. Inanami, M. Kuwabara, H. Harashima, and H. Kamiya.** 2006. Effects of overexpression and antisense RNA expression of Orf17, a MutT-type enzyme. Biol. Pharm. Bull. **29:**1087–1091.
35. **Jiang, D., Z. Hatahet, J. O. Blaisdell, R. J. Melamede, and S. S. Wallace.** 1997. *Escherichia coli* endonuclease VIII: cloning, sequencing, and overexpression of the *nei* structural gene and characterization of *nei* and *nei nth* mutants. J. Bacteriol. **179:**3773–3782.
36. **Kamiya, H.** 2003. Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/nitrogen species: approaches using synthetic oligonucleotides and nucleotides: survey and summary. Nucleic Acids Res. **31:**517–531.
37. **Kamiya, H., E. Iida, N. Murata-Kamiya, Y. Yamamoto, T. Miki, and H. Harashima.** 2003. Suppression of spontaneous and hydrogen peroxide-induced mutations by a MutT-type nucleotide pool sanitization enzyme, the *Escherichia coli* Orf135 protein. Genes Cells **8:**941–950.
38. **Kamiya, H., and H. Kasai.** 1995. Formation of 2-hydroxydeoxyadenosine triphosphate, an oxidatively damaged nucleotide, and its incorporation by DNA polymerases. Steady-state kinetics of the incorporation. J. Biol. Chem. **270:**19446–19450.
39. **Kamiya, H., N. Murata-Kamiya, E. Iida, and H. Harashima.** 2001. Hydrolysis of oxidized nucleotides by the *Escherichia coli* Orf135 protein. Biochem. Biophys. Res. Commun. **288:**499–502.
40. **Kasak, L., R. Hõrak, and M. Kivisaar.** 1997. Promoter-creating mutations in *Pseudomonas putida*: a model system for the study of mutation in starving bacteria. Proc. Natl. Acad. Sci. USA **94:**3134–3139.
41. **Kobayashi, M., Y. Ohara-Nemoto, M. Kaneko, H. Hayakawa, M. Sekiguchi, and K. Yamamoto.** 1998. Potential of *Escherichia coli* GTP cyclohydrolase II for hydrolyzing 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. J. Biol. Chem. **273:**26394–26399.
42. **Koch, B., L. E. Jensen, and O. Nybroe.** 2001. A panel of Tn*7*-based vectors for insertion of the *gfp* marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. J. Microbiol. Methods **45:**187–195.
43. **Lambertsen, L., C. Sternberg, and S. Molin.** 2004. Mini-Tn*7* transposons for site-specific tagging of bacteria with fluorescent proteins. Environ. Microbiol. **6:**726–732.
44. **Lea, D. E., and C. A. Coulson.** 1949. The distribution of the numbers of mutants in bacterial populations. J. Genet. **49:**264–265.
45. **Maki, H., and M. Sekiguchi.** 1992. MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. Nature **355:**273–275.
46. **Martinez, A., and R. Kolter.** 1997. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. J. Bacteriol. **179:**5188–5194.
47. **Matic, I., F. Taddei, and M. Radman.** 2004. Survival versus maintenance of genetic stability: a conflict of priorities during stress. Res. Microbiol. **155:**337–341.
48. **Matsumoto, Y., Q. M. Zhang, M. Takao, A. Yasui, and S. Yonei.** 2001. *Escherichia coli* Nth and human hNTH1 DNA glycosylases are involved in removal of 8-oxoguanine from 8-oxoguanine/guanine mispairs in DNA. Nucleic Acids Res. **29:**1975–1981.
49. **McKenzie, G. J., P. L. Lee, M. J. Lombardo, P. J. Hastings, and S. M. Rosenberg.** 2001. SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. Mol. Cell **7:**571–579.
50. **McLennan, A. G.** 2006. The Nudix hydrolase superfamily. Cell Mol. Life Sci. **63:**123–143.
51. **Michaels, M. L., C. Cruz, A. P. Grollman, and J. H. Miller.** 1992. Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. Proc. Natl. Acad. Sci. USA **89:**7022–7025.
52. **Michaels, M. L., and J. H. Miller.** 1992. The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). J. Bacteriol. **174:**6321–6325.
53. **Michaels, M. L., L. Pham, C. Cruz, and J. H. Miller.** 1991. MutM, a protein that prevents G · C—T · A transversions, is formamidopyrimidine-DNA glycosylase. Nucleic Acids Res. **19:**3629–3632.
54. **Michaels, M. L., J. Tchou, A. P. Grollman, and J. H. Miller.** 1992. A repair system for 8-oxo-7,8-dihydrodeoxyguanine. Biochemistry **31:**10964–10968.
55. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
56. **Nair, S., and S. E. Finkel.** 2004. Dps protects cells against multiple stresses during stationary phase. J. Bacteriol. **186:**4192–4198.
57. **Neeley, W. L., S. Delaney, Y. O. Alekseyev, D. F. Jarosz, J. C. Delaney, G. C. Walker, and J. M. Essigmann.** 2007. DNA polymerase V allows bypass of toxic guanine oxidation products *in vivo*. J. Biol. Chem. **282:**12741–12748.
58. **Neeley, W. L., and J. M. Essigmann.** 2006. Mechanisms of formation, genotoxicity, and mutation of guanine oxidation products. Chem. Res. Toxicol. **19:**491–505.
59. **Nghiem, Y., M. Cabrera, C. G. Cupples, and J. H. Miller.** 1988. The *mutY* gene: a mutator locus in *Escherichia coli* that generates G · C—T · A transversions. Proc. Natl. Acad. Sci. USA **85:**2709–2713.
60. **Oliver, A., R. Canton, P. Campo, F. Baquero, and J. Blazquez.** 2000. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science **288:**1251–1254.
61. **Oliver, A., J. M. Sanchez, and J. Blazquez.** 2002. Characterization of the GO system of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **217:**31–35.
62. **Pavel, H., M. Forsman, and V. Shingler.** 1994. An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of *Pseudomonas* sp. strain CF600 on para-substituted methylphenols. J. Bacteriol. **176:**7550–7557.
63. **Rosche, W. A., and P. L. Foster.** 2000. Determining mutation rates in bacterial populations. Methods **20:**4–17.
64. **Rosenberg, S. M.** 2001. Evolving responsively: adaptive mutation. Nat. Rev. Genet. **2:**504–515.
65. **Saito, Y., F. Uraki, S. Nakajima, A. Asaeda, K. Ono, K. Kubo, and K. Yamamoto.** 1997. Characterization of endonuclease III (*nth*) and endonuclease VIII (*nei*) mutants of *Escherichia coli* K-12. J. Bacteriol. **179:**3783–3785.
66. **Saumaa, S., K. Tarassova, M. Tark, A. Tover, R. Tegova, and M. Kivisaar.** 2006. Involvement of DNA mismatch repair in stationary-phase mutagenesis during prolonged starvation of *Pseudomonas putida*. DNA Repair **5:**505–514.
67. **Saumaa, S., A. Tover, L. Kasak, and M. Kivisaar.** 2002. Different spectra of stationary-phase mutations in early-arising versus late-arising mutants of *Pseudomonas putida*: involvement of the DNA repair enzyme MutY and the stationary-phase sigma factor RpoS. J. Bacteriol. **184:**6957–6965.
68. **Sharma, R. C., and R. T. Schimke.** 1996. Preparation of electrocompetent *E. coli* using salt-free growth medium. BioTechniques **20:**42–44.
69. **Shibutani, S., M. Takeshita, and A. P. Grollman.** 1991. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature **349:**431–434.
70. **Shimizu, M., P. Gruz, H. Kamiya, S. R. Kim, F. M. Pisani, C. Masutani, Y. Kanke, H. Harashima, F. Hanaoka, and T. Nohmi.** 2003. Erroneous incorporation of oxidized DNA precursors by Y-family DNA polymerases. EMBO Rep. **4:**269–273.
71. **Sokal, R. R., and F. J. Rohlf.** 1981. Biometry. W. H. Freeman, San Francisco, CA.
72. **Sung, H. M., G. Yeamans, C. A. Ross, and R. E. Yasbin.** 2003. Roles of YqjH and YqjW, homologs of the *Escherichia coli* UmuC/DinB or Y superfamily of DNA polymerases, in stationary-phase mutagenesis and UV-induced mutagenesis of *Bacillus subtilis*. J. Bacteriol. **185:**2153–2160.
73. **Sutton, M. D., and G. C. Walker.** 2001. Managing DNA polymerases: coordinating DNA replication, DNA repair, and DNA recombination. Proc. Natl. Acad. Sci. USA **98:**8342–8349.
74. **Tark, M., A. Tover, K. Tarassova, R. Tegova, G. Kivi, R. Hõrak, and M. Kivisaar.** 2005. A DNA polymerase V homologue encoded by TOL plasmid pWW0 confers evolutionary fitness on *Pseudomonas putida* under conditions of environmental stress. J. Bacteriol. **187:**5203–5213.
75. **Tegova, R., A. Tover, K. Tarassova, M. Tark, and M. Kivisaar.** 2004. Involvement of error-prone DNA polymerase IV in stationary-phase mutagenesis in *Pseudomonas putida*. J. Bacteriol. **186:**2735–2744.
76. **Timms, A. R., W. Muriel, and B. A. Bridges.** 1999. A UmuD,C-dependent pathway for spontaneous G:C to C:G transversions in stationary phase *Escherichia coli mut Y.* Mutat. Res. **435:**77–80.
77. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and the

methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. **120:**416–423.
78. **Wolf, S. G., D. Frenkiel, T. Arad, S. E. Finkel, R. Kolter, and A. Minsky.** 1999. DNA protection by stress-induced biocrystallization. Nature **400:**83–85.
79. **Xu, W., C. A. Dunn, F. O'Handley, S. D. L. Smith, and M. J. Bessman.** 2006. Three new Nudix hydrolases from *Escherichia coli*. J. Biol. Chem. **281:**22794–22798.
80. **Yamada, M., T. Nunoshiba, M. Shimizu, P. Gruz, H. Kamiya, H. Harashima, and T. Nohmi.** 2006. Involvement of Y-family DNA polymerases in mutagenesis caused by oxidized nucleotides in *Escherichia coli*. J. Bacteriol. **188:**4992–4995.
81. **Yanofsky, C., E. C. Cox, and V. Horn.** 1966. The unusual mutagenic specificity of an *E. coli* mutator gene. Proc. Natl. Acad. Sci. USA **55:**274–281.
82. **Yeiser, B., E. D. Pepper, M. F. Goodman, and S. E. Finkel.** 2002. SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness. Proc. Natl. Acad. Sci. USA **99:**8737–8741.
83. **Zhang, Q. M., N. Ishikawa, T. Nakahara, and S. Yonei.** 1998. *Escherichia coli* MutY protein has a guanine-DNA glycosylase that acts on 7,8-dihydro-8-oxoguanine:guanine mispair to prevent spontaneous G:C→C:G transversions. Nucleic Acids Res. **26:**4669–4675.

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Brief report

Involvement of DNA mismatch repair in stationary-phase mutagenesis during prolonged starvation of *Pseudomonas putida*

*Signe Saumaa, Kairi Tarassova, Mariliis Tark, Andres Tover, Radi Tegova, Maia Kivisaar**

Department of Genetics, Institute of Molecular and Cell Biology, Tartu University and Estonian Biocentre, 23 Riia Street, 51010 Tartu, Estonia

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ABSTRACT

One of the popular ideas is that decline in methyl-directed mismatch repair (MMR) in carbon-starved bacteria might facilitate occurrence of stationary-phase mutations. We compared the frequency of accumulation of stationary-phase mutations in carbon-starved *Pseudomonas putida* wild-type and MMR-defective strains and found that knockout of MMR system increased significantly emergence of base substitutions in starving *P. putida*. At the same time, the appearance of 1-bp deletion mutations was less affected by MMR in this bacterium. The spectrum of base substitution mutations which occurred in starving populations of *P. putida* wild-type strain was distinct from mutation spectrum identified in MMR-defective strains. The spectrum of base substitutions differed also in this case when mutants emerged in starved populations of MutS or MutL-defective strains were comparatively analyzed. Based on our results we suppose that other mechanisms than malfunctioning of MMR system in resting cells might be considered to explain the accumulation of stationary-phase mutations in *P. putida*. To further characterize populations of *P. putida* starved on selective plates, we stained bacteria with LIVE/DEAD kit in situ on agar plates. We found that although the overall number of colony forming units (CFU) did not decline in long-term-starved populations, these populations were very heterogeneous on the plates and contained many dead cells. Our results imply that slow growth of subpopulation of cells at the expenses of dead cells on selective plates might be important for the generation of stationary-phase mutations in *P. putida*. Additionally, the different survival patterns of *P. putida* on the same selective plates hint that competitive interactions taking place under conditions of prolonged starvation of microbial populations on semi-solid surfaces might be more complicated than previously assumed.

1. Introduction

Due to intense competition for nutrients, bacteria spend the majority of their lives under starvation conditions. Evolution occurs very rapidly in starved populations and takes advantage of different types of mutations. Apparently

* *Corresponding author*. Tel.: +372 7 375036; fax: +372 7 420286.
E-mail address: maiak@ebc.ee (M. Kivisaar).

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static microbial populations under the nonlethal selective pressure accumulate mutations called as adaptive mutations or stationary-phase mutations (reviewed in Refs. [1,2]). In addition to the appearance of mutants able to utilize novel growth substrates, starvation selects mutants with enhanced abilities to scavenge amino acids released from dead cells in bacterial populations (e.g. [3,4]). Cells with growth advantage in stationary-phase (GASP) phenotype either co-exist with the parental majority or displace the parent [3,5]. Foster [1] has pointed out that the rate of mutation under starvation conditions is too high to be accounted for by the amount of DNA synthesis if it is assumed that DNA synthesis in stationary-phase cells involves the whole genome and has an overall error rate similar to that of DNA replication in growing cells. However, some published reports [6,7] have implied that there was considerably more DNA synthesis under the starvation conditions than might have been assumed.

Starvation conditions encountered during stationary-phase incubation may permit a transient increase in the rate of mutation. Transient mutability induced by starvation has been shown in most natural isolates of *Escherichia coli* [8]. Also, results by Loewe et al. [9] suggest a correlation between deleterious mutation rate and the time bacteria spend in the stationary-phase. It is known, for example, that DNA damage, such as the formation of the 7,8-dihydro-8-oxoguanine (GO) can give rise to stationary-phase mutations [6,7,10]. Additionally, error-prone DNA polymerases including the members of DinB/UmuDC superfamily are implicated in stationary-phase mutations [11–15]. It is also possible that methyl-directed mismatch repair (MMR) might be disabled transiently during stationary-phase mutagenesis. This idea, although it has met controversy [16,17], was initially supported by the finding that the mutation spectrum observed in Lac^+ revertants emerging in a starving *E. coli* population was reproduced by MMR deficiency in growing cells [18]. Subsequent studies [19,20] demonstrated that MutS and MutH decline to levels appropriate for decreased DNA synthesis in stationary-phase *E. coli*, whereas functional MutL is limiting for MMR specifically during stationary-phase [20]. Some other studies, originally proposed by Schaaper and Radman [21], suggest that MMR deficiency is caused by saturation of the MMR system with an excess of DNA replication errors.

The genus *Pseudomonas* represents one of the largest groups of bacteria including both pathogenic and non-pathogenic species. Bacteria from the genus *Pseudomonas* are known for their ability to colonize multiple habitats and to adapt rapidly to new environments. The results of our recently published studies of stationary-phase mutagenesis in *Pseudomonas putida* suggest that mutation processes in cells that have been starved for a short period are not entirely compatible with those from a prolonged starvation [15,22]. In the current study we have investigated whether the mechanisms of occurrence of stationary-phase mutations in populations of long-term-starved *P. putida* could be explained by malfunctioning of MMR in this bacterium under starvation conditions. Our results indicated that MMR is not entirely disabled in starving *P. putida*. Specifically, we found that the frequency of accumulation of base substitution mutations was higher in MMR-deficient *P. putida* compared to the wild-type during the 2-week starvation period studied. Moreover, the spectrum of base substitutions which occurred in starved *P. putida* wild-type strain was distinct from that identified in MMR-defective strains. Characterization of populations of *P. putida* starved on selective plates by staining bacteria with LIVE/DEAD kit in situ on agar plates revealed that the ratio of living to dead cells varied regionally on the same plate whereas the proportion of dead cells increased with time of starvation. Hence, we suppose that the death of a subpopulation of cells under carbon starvation conditions allows slow growth of the other cells and this might be necessary for DNA replication and generation of mutations in long-term-starved populations of *P. putida*. Involvement of different stress-induced DNA polymerases in stationary-phase mutagenesis in *P. putida* will be also discussed.

2. Materials and methods

2.1. *Bacterial strains, plasmids, and media*

The bacterial strains and plasmids used in this study are described in Table 1. Complete medium was Luria–Bertani (LB) medium [23]. M9 minimal medium [24] was supplemented with solution of trace salts [25] at final concentration 2.5 ml/l. The content of this solution was following: 10.75 g MgO, 2 g $CaCO_3$, 4.5 g $FeSO_4{\cdot}7H_2O$, 1.44 g $ZnSO_4{\cdot}7H_2O$, 1.12 g $MnSO_4{\cdot}4H_2O$, 0.25 g $CuSO_4{\cdot}5H_2O$, 0.28 g $CoSO_4{\cdot}7H_2O$, and 0.06 g H_3BO_4 dissolved in 1 l water, supplemented by 51.3 ml concentrated HCl. Solid medium contained 1.5% Difco agar. Casamino acids (CAA) and glucose were added to the minimal medium at final concentrations 0.4% and 0.2%, respectively. Phenol minimal plates contained 2.5 mM phenol as a sole carbon and energy source. Antibiotics were added at the following concentrations: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml; tetracycline at 80 μg/ml; carbenicillin at 1000–3000 μg/ml and rifampin at 100 μg/ml. *E. coli* was incubated at 37 °C and *P. putida* at 30 °C. *E. coli* was transformed with plasmid DNA as described by Hanahan [26]. *P. putida* was electrotransformed as described by Sharma and Schimke [27]. *E. coli* strain TG1 [28] or DH5α (Invitrogen) was used for the DNA cloning procedures.

2.2. *Construction of P. putida DNA mismatch repair-deficient strains*

The part of the *mutS* gene of *P. putida* strain KT2440 lacking 796 nucleotides from 5′ end of the original 2571 nucleotide-long *mutS* (PP1626) was obtained from cosmid pMIR13415 [29]. The 5-kb BamHI-ClaI DNA fragment containing sequence of the *mutS* gene was cloned from cosmid pMIR13415 into the BamHI- and EcoRV-cleaved pBluescript SK (+) to obtain pSKrpoSmutS. The ClaI ends were blunt ended before the ligation. Then we deleted approximately 2.5-kb ClaI-EcoRI DNA fragment containing DNA sequences locating downstream of the *mutS* gene in pSKrpoSmutS to obtain pSKmutS. To interrupt the *mutS* sequence with antibiotic resistance gene, the Eco47III-cleaved DNA fragment containing the Km^r gene from pUTmini-Tn5 Km2 [30] was inserted into the SmaI-cleaved pSKmutS. The XbaI- and HincII-generated DNA fragment from pSKmutS::km was subsequently cloned into plasmid pGP704 L [31] by using XbaI and Ecl136II sites.

Table 1 – Bacterial strains and plasmids used for this study

Strain or plasmid	Genotype or construction	Source or reference
Strains		
E. coli		
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	[28]
DH5α	*supE44* Δ*lacU169*(f80 *lacZ*ΔM15) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Invitrogen
CC118 λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (Am) *recA1* λpir phage lysogen	[32]
P. putida		
PaW85	Wild type	[33]
PaWMutS	*mutS*::*km*	This study
PaWMutL	Δ*mutL*::*km*	This study
Plasmids		
pBluescript KS (+)	Cloning vector (Ap^r)	Stratagene
pBluescript SK (+)	Cloning vector (Ap^r)	Stratagene
pUTmini-Tn5 Km2	Delivery plasmid for mini-Tn5 Km2 (Ap^r Km^r)	[30]
pGP704 L	Delivery plasmid for homologous recombination (Ap^r)	[31]
pRK2013	Helper plasmid for conjugal transfer of pGP704 L (Km^r)	[34]
pMIR13415	Cosmid carrying partial sequence of the *mutS* gene from *P. putida*	[29]
pSKrpoSmutS	pBluescript SK (+) containing BamHI-ClaI fragment from pMIR13415 cloned into BamHI-EcoRV-cleaved vector	This study
pSKmutS	pSKrpoSmutS with deletion of 2.5-kb ClaI-EcoRI fragment	This study
pSKmutS::km	*mutS* in pSKmutS is interrupted with Km^r gene from pUTmini-Tn5 Km2	This study
pGP704mutS::km	pGP704 L with XbaI-HincII fragment of *mutS*::*km* from pSKmutS::km in XbaI-Ecl136II-cleaved vector	This study
pSKmutLlopp	pBluescript SK (+) containing PCR-amplified *mutL* DNA region encoding C-terminal part of MutL inserted as EcoRV-XhoI fragment	This study
pSKmutL	pSKmutLlopp containing PCR-amplified *mutL* DNA region encoding N-terminal part of MutL inserted as AviII-XhoI fragment in XhoI site	This study
pSKΔmutL::km	*mutL* in pSKmutL is interrupted with Km^r gene from pUTmini-Tn5 Km2 by replacing AatII-Bsp119I-generated fragment from *mutL* with Km^r gene	This study
pGP704ΔmutL::km	pGP704 L with XbaI-Bpu1102I fragment of Δ*mutL*::*km* from pSKΔmutL::km in EcoRV-cleaved vector	This study
pKTpheA56+A	Test system for detection of Phe$^+$ revertants occurring due to 1-bp deletions	[15]
pKTpheA22TGA	Test system for detection of Phe$^+$ revertants occurring due to base substitutions	[15]
pKTpheA22TAA	Test system for detection of Phe$^+$ revertants occurring due to base substitutions	[15]
pKTpheA22TAG	Test system for detection of Phe$^+$ revertants occurring due to base substitutions	[15]
pKTluxAB	pKT240-derivative carrying *luxAB* genes (Ap^r)	[15]

Plasmid pGP704mutS::km was selected in *E. coli* strain CC118 λpir [32]. The interrupted *mutS* gene was inserted into the chromosome of *P. putida* PaW85 [33] by homologous recombination. Plasmid pGP704mutS::km, not able to replicate in hosts other than *E. coli* CC118 λpir, was conjugatively transferred into *P. putida* PaW85 by using helper plasmid pRK2013 [34]. The PaW85 *mutS*::*km* knockout strain PaWMutS was verified by PCR analysis using primers PpmutSNdeI (5′-CATATGTCAGATCTTTCCGCACAC-3′), complementary to the sequences −3 to +21 relatively to the ATG initiator codon of the *mutS* gene, and KmOc (5′-TCGAGCAAGACGTTTCCC-3′), complementary to the sequences 34–16 nucleotides downstream of the ATG initiator codon of Km^r gene, respectively. Additionally, we could confirm that the spontaneous frequency of mutation to rifampin resistance was about 1000-fold higher in MutS-defective bacteria compared to the wild-type strain.

The *mutL* (PP4896) gene sequence of *P. putida* KT2440 was obtained from The Institute for Genomic Research website (http://www.tigr.org). The *mutL* gene was amplified by PCR from genomic DNA of *P. putida* PaW85, which is isogenic to *P. putida* strain KT2440. Four primers were used for the amplification of the *mutL* gene. Firstly, two primers, MutLloppRev (5′-GCCGGTGCGGGTGGGCAGCG-3′), complementary to the sequence +193 to +174 downstream of the TGA stop codon and MutLloppFW (5′-GGCACCTTGCACCGTGCCGTT-3′), complementary to the sequence +978 to +998 nucleotides downstream of the ATG initiator codon of the *mutL* gene, were used to amplify 1125-nucleotide DNA region of the 1896 nucleotide-long *mutL* gene. The PCR product was cleaved with EcoRV and XhoI enzymes generating 905-bp DNA fragment which was subsequently subcloned into EcoRV- and XhoI-cleaved pBluescript SK (+), to obtain pSKmutLlopp. The second half of the *mutL* gene was amplified using two primers, MutLalgus-Rev (5′-GTCGCCCTGGCTTTCGGGCAG-3′) complementary to the sequence +1313 to +1293 downstream of the ATG initiator codon and MutLalgusFW (5′-ACCGTACGCCCTGGCGAAACC-3′) complementary to the sequence −149 to −129 upstream of the ATG initiator codon of the *mutL* gene. The amplified 1500-bp DNA fragment containing the second half of the *mutL* gene was cleaved with AviII and XhoI, and the obtained 1212-bp DNA fragment was inserted into XhoI-cleaved pSKmutLlopp resulting in plasmid pSKmutL. The Km^r gene was amplified by PCR from plasmid pUTmini-Tn5 Km2 by using the primer KmSac [35]. The Ecl136II-cleaved DNA fragment containing the Km^r gene was used to replace the AatII- and Bsp119I-generated 880-bp fragment in the *mutL* gene in plasmid pSKmutL.

The resulting ΔmutL::km sequence from pSKΔmutL::km was inserted into EcoRV-cleaved plasmid pGP704 L by using XbaI and Bpu1102I sites. The XbaI and Bpu1102I ends were blunt ended before the ligation. Plasmid pGP704ΔmutL::km was selected in *E. coli* strain CC118 λpir. The interrupted *mutL* gene containing the internal deletion was inserted into the chromosome of *P. putida* PaW85 by homologous recombination. Plasmid pGP704ΔmutL::km, not able to replicate in hosts other than *E. coli* CC118 λpir, was conjugatively transferred into *P. putida* PaW85 by using helper plasmid pRK2013. The PaW85 ΔmutL::km knockout strain PaWMutL was verified by PCR analysis using primers KmOc and MutLalgusFW and by measuring the spontaneous frequency of mutation to rifampin resistance. Similarly to the PaWMutS, the frequency of mutation of Rif^r was increased about 1000-fold in PaWMutL compared to the wild-type strain.

2.3. Isolation and analysis of Phe^+ revertants

Test systems used for detection of different point mutations were the same as previously described [15]. When phenol monooxygenase *pheA*-expressing plasmid is introduced into phenol-nondegrading *P. putida* strain PaW85 lacking this gene in a chromosome, bacteria gain the ability to utilize phenol as a sole carbon source. Sequence of the reporter gene *pheA* encoding phenol monooxygenase was altered in RSF1010-derived tester plasmids either by +1 frameshift mutation (plasmid pKTpheA56+A) or by introducing different translational stop codons into the same position (Leu-22) of the *pheA* sequence (plasmids pKTpheA22TAA, pKTpheA22TGA, or pKTpheA22TAG). Incubation of *P. putida* strain carrying any of these tester plasmids with mutated *pheA* sequence on minimal selective plates containing phenol as an only carbon source enables to isolate phenol-growing (Phe^+) revertants. Independent cultures of *P. putida* strains carrying different tester plasmids for the detection of Phe^+ revertants [15] were generated by growing cells to late logarithmic growth phase in M9 medium containing glucose and CAA, diluting this culture by 10^5 into fresh glucose and CAA-containing M9 medium, dispensing 2-ml aliquots into test tubes and allowing cells to reach saturation by growing cells for 18–20 h. Cells sampled from the culture were harvested by centrifugation and washed in M9 solution. Approximately 5×10^8 cells of *P. putida* wild-type and 2×10^8 cells of MMR-defective strains with test system measuring frameshift mutations, or 2×10^7 cells of MMR-defective strains carrying test systems allowing detection of base substitution mutations were spread onto phenol minimal plates containing 1500 μg/ml carbenicillin. When fewer amounts of cells of the tester strains were plated onto selective plates, they were plated with approximately 5×10^8 scavenger cells. Scavenger cells (*P. putida* PaW85 carrying plasmid pKTluxAB [15]) were grown to saturation in M9 minimal medium containing glucose and CAA. In order to avoid inhibition of accumulation of mutants caused by the presence of earlier-emerged mutant colonies on selective plates [22], all counted colonies were removed, as soon as they appeared, by cutting them off on small agar plugs. We have previously shown [15,36] that Phe^+ colonies appearing on phenol minimal plates on day 2 contained mutations that occurred before the plating in a growing culture, whereas colonies that emerged on selective plates on day 3 and later contained mutations that occurred after the cells were plated. The latter were called as stationary-phase mutations. Based on our previous studies [15,36] we can confirm that the copy number of the tester plasmids is not affected by the growth phase of the bacteria. Here, we addressed the same question by comparing the number of the plasmid copies in MMR-defective and wild-type strains but no differences were found.

In Phe^+ mutants, an approximately 350-bp DNA region covering the area of the *pheA* gene containing potential reversion mutations was analyzed by DNA sequencing as described previously [15]. The DNA sequencing reactions were analyzed with an ABI Prism 377 DNA Sequencer (Perkin-Elmer).

2.4. Measurement of number of colony forming units (CFU) in starving *P. putida* cultures incubated on phenol minimal plates

In order to control whether bacteria would die during starvation on the selective medium, we measured the number of colony forming units (CFU) for at least five independent starving cultures of wild-type or MMR-defective strains. The viability of bacteria was determined on the same plates that were used for the isolation of Phe^+ revertants. Using sterile 1-ml pipette tips, small plugs were cut out from the phenol-containing minimal plates avoiding Phe^+ colonies. Bacteria from the plugs were suspended in M9 solution by shaking. Thereafter, appropriate dilutions were made and plated onto LB plates to determine the number of CFU. The similar results were obtained despite CFU determinations were performed by plating bacteria onto LB or M9 minimal glucose medium in the presence or absence of carbenicillin. As the resistance to carbenicillin is provided by plasmids carrying the test systems for detection of point mutations, these results confirm that the tester plasmids are stably maintained in cells survived in *P. putida* starving populations.

2.5. Cell staining and microscopic examination of *P. putida* starved populations on phenol minimal plates

We cut approximately 1 cm^2 agar pieces containing starved *P. putida* cells from phenol-containing selective plates and placed these pieces onto a glass slide. Thereafter, we added 10 μl of diluted LIVE/DEAD *Bac*Light 7012 kit (Molecular Probes, Inc., Eugene, OR, USA) stain mixture of SYTO 9 and propidium iodide at final concentrations of 10 and 60 μM, respectively, onto the surface of the agar containing the cell lawn and covered gently with coverslip. The samples were incubated with stain mixture in the dark for 10 min at room temperature. Stained cell populations were observed under an Olympus BX 41 epifluorescent microscope equipped with a mercury lamp and with the appropriate filters. The samples were excited at 470 nm allowing simultaneous viewing red and green fluorescent cells. Green fluorescent cells were considered to be alive; other cells were considered to be dead [37]. In all cases, a 100× objective was used with immersion oil, giving a total magnification of 1000×. Images were captured with an Olympus U-TV0.5XC camera and were processed with DP Controller 1.2.1. 108 and DP Manager 1.2.1. 107. A minimum of 20 fields selected at random were examined on each sample.

3. Results and discussion

3.1. *Mismatch repair is involved in avoidance of stationary-phase mutations both in growing and starving populations of P. putida*

One of the widely disputed ideas is that MMR is active and essential for mutation avoidance in growing bacteria but its malfunctioning in starving cells might facilitate occurrence of stationary-phase mutations [16,17]. Here, we studied whether the occurrence of stationary-phase mutations in *P. putida* could be explained by malfunctioning of MMR during prolonged starvation of bacteria. For that purpose, we constructed *P. putida* MMR-defective strains PaWMutS and PaWMutL either defective in MutS or MutL and compared the frequency of occurrence of mutants in these strains with that in the wild-type strain PaW85. The test systems used for the selection of mutants measured appearance of phenol-degrading (Phe^+) revertants as previously described [15]. No differences were observed in viability between the wild-type strain PaW85 and MMR-deficient strains PaWMutS and PaWMutL even during prolonged incubation of bacteria under starvation conditions, and the number of CFU did not decline in starving populations (Fig. 1A).

The results presented in Fig. 1B show the accumulation of the Phe^+ revertants which occurred in starving populations of *P. putida* wild-type strain and MMR-defective strains due to base substitution mutations eliminating TAG stop codon. The similar accumulation curves appeared when we used two other assay systems detecting changes removing either TAA or TGA stop codon (data not shown). Base substitution mutants accumulated in *P. putida* wild-type strain at well-detectable level already at the beginning of starvation and the frequency of appearance of these revertants remained constant during the 2-week-starvation period studied. The similar accumulation dynamics of the base substitution mutants became evident also with MMR-defective strains PaWMutS and PaWMutL (Fig. 1B). At the same time, the frequency of accumulation of base substitution mutants was approximately 25 times elevated in starved populations of PaWMutS and PaWMutL strains compared to the wild-type strain throughout all starvation period studied (Fig. 1B). This indicated that the efficiency of MMR in repairing base substitutions did not decline with time of starvation of *P. putida*.

We have previously shown that the frequency of accumulation of 1-bp deletion mutants significantly increased in long-term-starved *P. putida* populations [15]. The results presented in Fig. 1C demonstrated that during the first 7 days of carbon starvation, the absence of MutL or MutS in *P. putida* increased the frequency of accumulation of 1-bp deletants approximately four and seven times, respectively, compared to the wild-type populations. Similarly to the wild-type strain, the frequency of appearance of the Phe^+ revertants increased significantly with time of starvation in MMR-defective strains as well. For example, during the shorter period of starvation (days 3–7) in average 0.44 deletion mutants appeared per day per 5×10^8 cells of MutS-defective strain, whereas on longer starvation period of PaWMutS (days 11–15) the frequency of accumulation of the revertants was elevated 10 times (in average 4.2 mutants per day per 5×10^8 cells). The remarkable increase in the rate of the accumulation of the revertants (about 15-fold) appeared also in this case when we used PaWMutL. At the same time, these results demonstrated that the lack of the MMR system has less influence upon rate of the occurrence of 1-bp deletion mutants compared to the emergence of base substitution mutants in starving *P. putida* populations (compare Fig. 1B and C). Moreover, if we compared

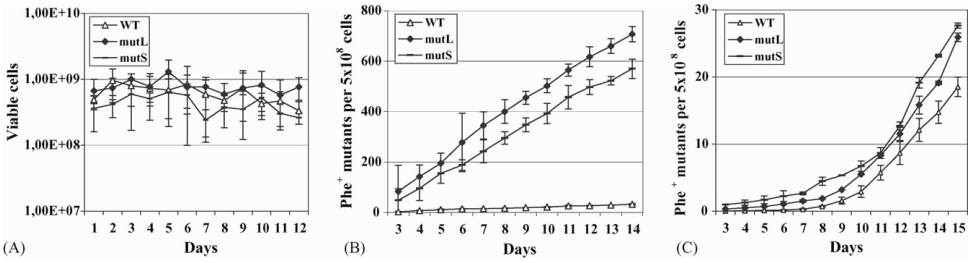

Fig. 1 – (A) Viability of *P. putida* wild-type strain PaW85 (WT), and its MMR-defective derivatives PaWMutS (mutS) and PaWMutL (mutL) on phenol minimal plates. Mean ± standard deviation (error bars) for at least five independent determinations are shown. 1.0E + 08, for example, indicates 10^8 viable cells. (B) Accumulation of Phe^+ revertants on phenol minimal plates in *P. putida* wild-type strain PaW85, and in its MMR-defective derivatives PaWMutS and PaWMutL carrying the tester plasmid pKTpheA22TAG which measures elimination of TAG stop codon by the base substitutions within the *pheA* gene. About 5×10^8 *P. putida* wild-type cells or 2×10^7 MMR-defective cells with 5×10^8 scavenger cells (PaW85 carrying pKTluxAB) were plated from overnight in liquid M9 medium-grown independent cultures onto phenol minimal plates. Such amounts of tester plasmid-carrying cells produced no more than two colonies per plate per day in average. (C) Accumulation of Phe^+ mutants on phenol minimal plates in *P. putida* wild-type strain PaW85, and in its MMR-defective derivatives carrying the tester plasmid pKTpheA56+A which measures reversion of +1 frameshift within the *pheA* gene. About 5×10^8 *P. putida* wild-type cells or 2×10^8 MMR-defective cells with 5×10^8 scavenger cells (PaW85 carrying pKTluxAB) were plated from overnight in liquid M9 medium-grown independent cultures onto phenol minimal plates. In all cases, mean ± standard deviations (error bars) for at least 10 plates calculated per 5×10^8 are shown.

the frequency of accumulation of 1-bp deletion mutations in the MMR-defective strains to that in the wild-type on day 10 and later, we did not detect remarkable differences at all. It is possible that different types of errors are corrected by MMR with different efficiency in starving *P. putida*. Also, our previous results demonstrated that *dinB* (PP1203)-encoded Pol IV homologue is required for most 1-bp deletions detected in long-term-starved populations of *P. putida* [15]. As the frequency of accumulation of the revertants increased with time of starvation also in MMR-defective strains, this data implied that the increased accumulation rate of the DinB-dependent mutations in the long-term-starved *P. putida* wild-type populations might be (at least partially) ascribed to increase in the activity and/or amount of DinB. At the same time, some studies, originally proposed by Schaaper and Radman [21], suggest that MMR deficiency is caused by saturation of the MMR system with an excess of DNA replication errors. Indeed, the results by Wagner and Nohmi [38] have demonstrated saturation of the MMR system as a result of accumulation of errors made by overproduction of Pol IV in *E. coli*. The spectrum of stationary-phase mutations of Lac$^+$ revertants in *E. coli* FC40 system which also measures frameshift mutations resembled mutations that appear in MMR-deficient cells [18], whereas Pol IV was required specifically for 1-bp deletions in stationary-phase cells but not in growing cells [12,13]. Hence, drawing parallels with these reports, and comparing the results presented herein we cannot exclude the possibility that the appearance of 1-bp deletion mutants in long-term-starved *P. putida* wild-type strain is in some extent facilitated by partial titration of MMR due to increase in DinB-caused replication errors in starving *P. putida*.

3.2. *Spectrum of base substitution mutations is affected by growth phase of P. putida and the presence of DNA repair functions in bacteria*

In order to study whether and how the spectrum of base substitution mutations could be affected by the activity of MMR enzymes MutS and MutL in *P. putida* and whether the growth phase of bacteria could influence the frequency of occurrence of different nucleotide changes, we analyzed the phenol monooxygenase gene *pheA* sequence of the Phe$^+$ revertants either collected from growing or stationary-phase populations of the wild-type and MMR-defective strains. The results of the DNA sequence analysis of the Phe$^+$ mutants which occurred due to elimination of TAG, TAA or TGA stop codons are summarized in Table 2. The T-to-C transition was the most prominent change in all cases studied. However, the spectra of mutations which eliminated either TGA or TAG stop codon in the *pheA* gene were more heterogeneous than the spectrum of substitutions which resulted in elimination of TAA codon. Although the usage of TAA codon did not allow detection of A-to-G transitions due to the generation of other stop codons TGA or TAG, we did not notice other changes (e.g., substitutions T-to-A; A-to-C; A-to-T) that potentially could also eliminate this stop codon.

The spectra of base substitutions identified among revertants isolated in growing cultures were distinct from those characterized for mutants accumulated in starving populations. These spectra were also dependent on the sequence of the stop codon used in the particular assay system. In the wild-type strain, the mutants isolated in growing cultures revealed the most heterogeneous spectrum of changes with the test system which measured the elimination of TGA stop codon. At the same time, if bacteria were starved, the TAG codon was eliminated with wider spectrum of mutations than the elimination of TGA or TAA codons.

The spectra of the base substitutions identified in starving populations of the wild-type strain were distinct from those characterized in MMR-defective strains. Presumably, if the MMR system is non-functional in starving bacteria, then the spectrum of mutations described in the starving wild-type strain should resemble spectrum observed in the MMR-defective strains. We can confirm that the mutation spectra characterized in the wild-type and MMR-defective strains differed from each other also in this case if mutants emerged in more than 1-week-starved populations were analyzed separately from the earlier arisen mutants (data not shown). Thus, the results of the analysis of spectra of base substitution mutations in different genetic backgrounds support the idea that the MMR system is functional at least during 2-week carbon starvation period in stationary-phase *P. putida*. Both types of base substitutions, transversions and transitions, were represented among the Phe$^+$ revertants which were isolated in the wild-type *P. putida* populations. However, the revertants which were collected in populations of MMR-defective strains contained mostly two types of transitions, T-to-C or A-to-G. Thus, similarly to *E. coli* MMR system [39], the MMR pathway in *P. putida* preferentially avoids transition mutations.

Surprisingly, spectrum of mutations which restored the functional *pheA* sequence in MMR-defective bacteria was also dependent on which component of MMR was inactivated. This difference appeared only in this case when we characterized stationary-phase mutations. The dissimilarity was the most significant between the spectra of mutations which eliminated the TAG stop codon in the *pheA* coding sequence (Table 2). In the MutS-defective strain the proportion of the A-to-G transitions increased from 18% in growing cells to 70% in starved populations, but the change was less significant when the revertants collected in the MutL defective strain were analyzed (14% A-to-G transitions in growing cells versus 33% in starved bacteria). One possible explanation of this phenomenon is that the absence of MutS or MutL in cells might influence differently DNA replication. Data from the literature indicate that different DNA polymerases may create different spectra of mutations within the same DNA sequence [40]. In *E. coli* it has been shown that the β processivity clamp of the replicative DNA polymerase Pol III communicates directly with multiple proteins to promote DNA replication and DNA repair [41]. Besides interacting with different DNA polymerases, *E. coli* β processivity clamp binds the MMR protein MutS and ligase [42]. The finding that the clamp loader and all the polymerases present in *E. coli* interact with the β clamp at the same location has suggested that clamp binding may be competitive and regulated [43]. A pentapeptide motif QL[SD]LF which is sufficient to enable interaction of these proteins with an archetypical *E. coli* β clamp is present in most sequenced members of eubacterial DNA polymerases and MutS proteins [44]. As *P. putida* MutS protein also contains this motif, it is very likely that the MutS protein interacts with β clamp in this organism. Thus, one may

Table 2 – Reversion of different nonsense mutations (TGA, TAA, TAG) in Phe$^+$ mutants accumulating in *P. putida* wild-type strain and its MutS- and MutL-defective derivatives[a]

Target[b]	DNA change	Occurrences						Reversion	Amino acid change
		Wild type growing cells	Starving cells	*mutS* Growing cells	Starving cells	*mutL* Growing cells	Starving cells		
TGA	T → C	38 (69%)	13 (54%)	65(92%)	117 (76%)	25 (93%)	82 (81%)	CGA	Arg
	T → G	3 (5.5%)	5 (21%)	0	0	0	3 (3%)	GGA	Gly
	T → A	1 (2%)	0	0	0	0	1 (1%)	AGA	Arg
	G → C	4 (7%)	0	0	0	0	0	TCA	Ser
	G → T	3 (5.5%)	4 (17%)	0	0	0	0	TTA	Leu
	A → C	4 (7%)	2 (8%)	3 (4%)	0	0	0	TGC	Cys
	A → G	0	0	3 (4%)	37 (24%)	2 (7%)	15 (15%)	TGG	Trp
	A → T	2 (4%)	0	0	0	0	0	TGT	Cys
TAA	T → C	56 (100%)	38 (88%)	63 (100%)	144 (99%)	17 (100%)	114 (100%)	CAA	Gln
	T → G	0	5 (12%)	0	1 (1%)	0	0	GAA	Glu
TAG	T → C	48 (98%)	44 (61%)	60 (82%)	26 (28%)	18 (86%)	62 (67%)	CAG	Gln
	T → G	0	6 (8.5%)	0	0	0	0	GAG	Glu
	T → A	0	1 (1.5%)	0	0	0	0	AAG	Lys
	G → C	0	1 (1.5%)	0	0	0	0	TAC	Tyr
	G → T	0	8 (11%)	0	0	0	0	TAT	Tyr
	A → C	0	0	0	2 (2%)	0	0	TCG	Ser
	A → G	0	11 (15%)	13 (18%)	66 (70%)	3 (14%)	30 (33%)	TGG	Trp
	A → T	1 (2%)	1 (1.5%)	0	0	0	0	TTG	Leu

[a] The spectrum of base substitutions occurring in growing bacteria was derived from analysis of DNA sequence of Phe$^+$ revertants appeared onto selective plates on day 2. Mutant colonies used for identification of stationary-phase mutations were picked up on days 3–12. Approximately 10–15 mutants were analyzed per each day. We did not notice remarkable changes in the spectrum of mutations in revertants either derived from earlier or later period of starvation (days 3–7 or days 8–12, respectively).

[b] In all cases the same codon, CTG for Leu 22, was altered in the wild-type *pheA* sequence.

hypothesize that the absence of MutS may somehow influence competitive interactions of different DNA polymerases with the β clamp.

In *E. coli*, the involvement of Pol V in stationary-phase mutagenesis has demonstrated in assaying reversion of base pair substitutions [11] but not in test systems that measure frameshift mutations [11-13]. Unlike *E. coli*, *Pseudomonas* species do not harbor chromosomally encoded Pol V. Instead, the genome of *P. putida* like the vast majority of recently sequenced bacterial genomes carries a second copy of the *dnaE* gene (PP3119) (http://www.tigr.org). The deduced amino acid sequence of this gene exhibits 30% identity to the deduced amino acid sequence of *dnaE* gene PP1606 encoding catalytic subunit of Pol III. Here we decided to name this *dnaE* homologue (PP3119) in *P. putida* as *dnaE2*. In the pathogen *Mycobacterium tuberculosis* DnaE1 is the replicative polymerase but DnaE2 mediates SOS mutagenesis and contributes to the emergence of drug resistance in vivo [45]. In *Caulobacter crescentus* an operon composed of two hypothetical genes and *dnaE2* is damage-inducible in a *recA*-dependent manner and is responsible for most DNA damage-induced mutations [46]. This widespread operon has been shown to be regulated by LexA also in *P. putida* [47]. Our preliminary results (not shown in this paper) have demonstrated that inactivation of *dnaE2* in MutS-defective strain resulted in reduction in the fraction of A-to-G transitions to the level similar to that observed in MutL-defective strain PaWMutL. Therefore, it is possible that DnaE2 might be involved in stationary-phase mutagenesis, this polymerase may create different spectrum of mutations than other DNA polymerase(s) and MutS may somehow suppress access of DnaE2 to the replication apparatus. Further studies are necessary to examine this intriguing possibility.

3.3. *Starving populations of P. putida are highly heterogeneous*

Our results presented above demonstrated that the frequency of accumulation of stationary-phase mutations was higher in MMR knockout strains PaWMutS and PaWMutL compared to the wild-type strain. According to the model that only a few cells in a stationary-phase population differentiate into a hypermutable state [48–51] one may argue that MMR becomes limiting only in a small subpopulation and therefore the overall frequency of mutations in starving wild-type population cannot be as high as in experiments performed with MMR-defective strains. However, the facts that the spectra of base substitution mutations characterized in starving populations of *P. putida* wild-type strain were distinct from those identified in the MMR-defective strains and that the frequency of frameshift mutations increased with time of starvation also in MMR-defective strains do not support this idea. Thus, the occurrence of stationary-phase mutations in *P. putida* cannot be explained simply by malfunctioning of MMR in long-term-starved bacteria.

To further characterize starving *P. putida* populations on phenol selective plates, we stained bacteria in situ on agar medium with LIVE/DEAD kit and visualized cells by epifluorescent microscopy using a 1000-fold magnification. The staining kit contains two nucleic acid stains and distinguishes live bacterial cells from dead by means of membrane integrity. The

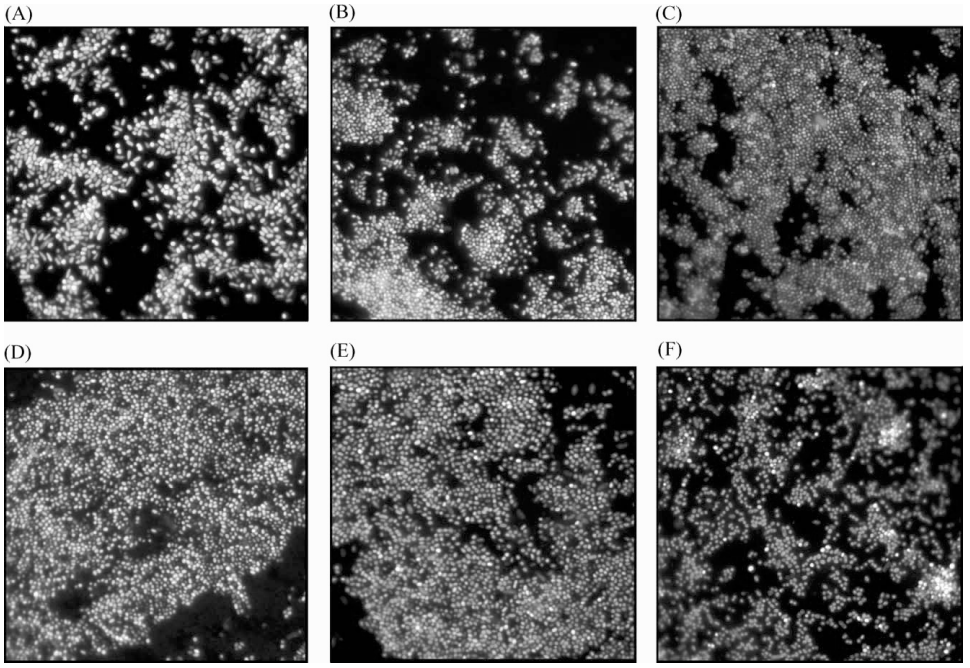

Fig. 2 – Characterization of *P. putida* populations on phenol minimal plates. Bacteria were stained with the LIVE/DEAD kit and visualized by epifluorescent microscopy using 1000-fold magnification. Samples are taken at different time points. (A) Cells immediately after plating; (B) and (C) 1-day-starved populations; (D), (E) and (F) 1-week-starved populations.

green fluorochrome (SYTO 9) is a small molecule that can penetrate intact plasma membranes while the larger red fluorochrome propidium iodide penetrates only compromised membranes. Bacterial suspensions incubated in the two stains simultaneously and then excited at 470 nm contain red and green fluorescent cells, depending on whether the bacteria are dead or live [37]. The most representative images of *P. putida* populations stained in situ on selective plates with LIVE/DEAD kit at different stages of starvation are shown in Fig. 2. Fig. 2A shows the image of *P. putida* wild-type cells that were stained directly after plating of 5×10^8 cells from stationary-phase liquid cultures onto phenol minimal plates. In this case, the majority of cells were green-colored. However, a small fraction of the cells was colored red which indicated that the plated bacterial populations contained some dead cells as well. During the next days of incubation of bacteria on selective plates the starving populations became significantly heterogeneous. The differences appeared already in this case when we examined 1-day-starved populations. The proportion of red-colored cells was increased in all studied fields. However, some fields contained more red-colored cells than the others (Fig. 2B and C) which indicated that cells incubated at the same selective plate may reside in different microenvironments. Visualization of *P. putida* populations starved for 1 week on phenol-containing minimal agar plates revealed the presence of many red-colored cells in populations. However, the ratio of green-to red-colored cells varied regionally on the same plate (Fig. 2D–F). Based on such variation, three different sub-classes were distinguished. Fig. 2D represents fields that mostly contained green-colored cells and red-colored cells were in minority. Fig. 2F depicts an opposite situation where majority of cells were red-colored. An intermediate survival pattern is visualized in Fig. 2E. Screening at random at least 20–30 fields on the same sample revealed that these three sub-classes were almost equally represented. The similar heterogeneity appeared by visualization of *P. putida* populations when we stained 2-week-starved cells or when we examined starved populations of MMR-defective strains (data not shown). At the same time, as already noted above (Fig. 1A), the number of CFU did not decline in *P. putida* populations with time of starvation. This discrepancy is difficult to explain. One may speculate that just after the plating bacteria can multiply a few generations on selective plates but this remains undetected because a fraction of cells which are colored green at the beginning of starvation are metabolically active but unable to form colony. Later, with time of starvation membranes of these cells become more compromised, these cells die and are colored red. Further experiments are needed to evaluate this intriguing possibility. However, the presence of many dead cells in long-term-starved population implies that a fraction of

cells in *P. putida* starving populations can grow at the expenses of nutrients released from the dead ones. Hence, we suppose that slow growth of fraction of cells might be necessary for DNA replication and generation of mutations in long-term-starved populations of *P. putida*.

Populations of stationary-phase cells have been shown to be highly dynamic: waves of fitter mutants constantly arise and take over previous populations [52]. The growth advantage in stationary-phase (GASP) phenotype has been observed in many different bacterial species [4,52]. So far, the appearance of GASP mutants has been studied in homogeneous liquid media. However, in natural habitats bacteria are living in a structured environment: they usually grow as biofilms, organised communities of cells embedded in an extracellular polysaccharide matrix and attached to a surface [53]. Our results of in situ staining of bacteria on selective plates (Fig. 2) provide insight into the processes of development of heterogeneity in starving microbial populations on semi-solid surface. We have noticed unequal distribution of living and dying cells in the different regions on the same selective plate. This implies that in the structured environment (e.g., on agar plates), the dying of bacteria may not occur randomly but is stimulated under certain conditions provided by a local environment. It is also possible that the emergence of individual GASP mutants on semi-solid surface may locally affect living conditions of the remaining cells, leading to different survival patterns at territory of the whole population. Thus, competitive interactions taking place in microbial populations might be more complicated than are currently appreciated.

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REFERENCES

[1] P.L. Foster, Adaptive mutation: the uses of adversity, Annu. Rev. Microbiol. 47 (1993) 467–504.
[2] P.L. Foster, Mechanisms of stationary phase mutation: a decade of adaptive mutation, Annu. Rev. Genet. 33 (1999) 57–88.
[3] M.M. Zambrano, D.A. Siegele, M. Almiron, A. Tormo, R. Kolter, Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures, Science 259 (1993) 1757–1760.
[4] E.R. Zinser, R. Kolter, *Escherichia coli* evolution during stationary phase, Res. Microbiol. 155 (2004) 328–336.
[5] B. Yeiser, E.D. Pepper, M.F. Goodman, S.E. Finkel, SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 8737–8741.
[6] B.A. Bridges, Elevated mutation rate in *mutT* bacteria during starvation: evidence for DNA turnover? J. Bacteriol. 178 (1996) 2709–2711.
[7] B.A. Bridges, S. Ereira, DNA synthesis and viability of a *mutT* derivative of *Escherichia coli* WP2 under conditions of amino acid starvation and relation to stationary-phase (adaptive) mutation, J. Bacteriol. 180 (1998) 2906–2910.
[8] I. Bjedov, O. Tenaillon, B. Gérard, V. Souza, E. Denamur, M. Radman, F. Taddei, I. Matic, Stress-induced mutagenesis in bacteria, Science 300 (2003) 1404–1409.
[9] L. Loewe, V. Textor, S. Scherer, High deleterious genomic mutation rate in stationary phase of *Escherichia coli*, Science 302 (2003) 1558–1560.
[10] B.A. Bridges, Spontaneous mutation in stationary-phase *Escherichia coli* WP2 carrying various DNA repair alleles, Mutat. Res. 302 (1993) 173–176.
[11] S. Bhamre, B.B. Gadea, C.A. Koyama, S.J. White, R.G. Fowler, An aerobic *recA*, *umuC*-dependent pathway of spontaneous base-pair substitution mutagenesis in *Escherichia coli*, Mutat. Res. 473 (2001) 229–247.
[12] H.J. Bull, M.-J. Lombardo, S.M. Rosenberg, Stationary-phase mutation in the bacterial chromosome: recombination protein and DNA polymerase IV dependence, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 8334–8341.
[13] G.J. McKenzie, P.L. Lee, M.-J. Lombardo, P.J. Hastings, S.M. Rosenberg, SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification, Mol. Cell 7 (2001) 571–579.
[14] H.-M. Sung, G. Yeamans, C.A. Ross, R.E. Yasbin, Roles of YgjH and YgjW, homologs of the *Escherichia coli* UmuC/DinB or Y superfamily of DNA polymerases, in stationary-phase mutagenesis and UV-induced mutagenesis of *Bacillus subtilis*, J. Bacteriol. 185 (2003) 2153–2160.
[15] R. Tegova, A. Tover, K. Tarassova, M. Tark, M. Kivisaar, Involvement of error-prone DNA polymerase pol IV on stationary phase mutagenesis in *Pseudomonas putida*, J. Bacteriol. 186 (2004) 2735–2744.
[16] P.L. Foster, Are adaptive mutations due to decline in mismatch repair? The evidence is lacking, Mutat. Res. 436 (1999) 179–184.
[17] R.S. Harris, G. Feng, K.J. Ross, R. Sidhu, C. Thulin, S. Longerich, S.K. Szigety, P.J. Hastings, M.E. Winkler, S.M. Rosenberg, Mismatch repair is diminished during stationary-phase mutation, Mutat. Res. 437 (1999) 51–60.
[18] S. Longerich, A.M. Galloway, R.S. Harris, C. Wong, S.M. Rosenberg, Adaptive mutation sequences reproduced by mismatch repair deficiency, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 12017–12020.
[19] G. Feng, H.-C.T. Tsui, M.E. Winkler, Depletion of the cellular amounts of the MutS and MutH methyl-directed mismatch repair proteins in stationary-phase *Escherichia coli* K-12 cells, J. Bacteriol. 178 (1996) 2388–2396.
[20] R.S. Harris, G. Feng, K.J. Ross, R. Sidhu, C. Thulin, S. Longerich, S.K. Szigety, M.E. Winkler, S.M. Rosenberg, Mismatch repair protein MutL becomes limiting during stationary-phase mutation, Genes Dev. 11 (1997) 2426–2437.
[21] R.M. Schaaper, M. Radman, The extreme mutator effect of *Escherichia coli mutD5* results from saturation of mismatch repair by excessive DNA replication errors, EMBO J. 8 (1989) 3511–3516.
[22] S. Saumaa, A. Tover, L. Kasak, M. Kivisaar, Different spectra of stationary-phase mutations in early-arising versus late-arising mutants of *Pseudomonas putida*: involvement of the DNA repair enzyme MutY and stationary-phase sigma factor RpoS, J. Bacteriol. 184 (2002) 6957–6965.
[23] J.H. Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[24] M.H. Adams, Bacteriophages, Interscience Publishers Inc., New York, 1959, pp. 445–447.
[25] T. Bauchop, S.R. Elsden, The growth of micro-organisms in relation to their energy supply, J. Gen. Microbiol. 23 (1960) 457–469.

[26] D. Hanahan, Studies on the transformation of *E. coli* with plasmids, J. Mol. Biol. 166 (1983) 577–580.
[27] R.C. Sharma, R.T. Schimke, Preparation of electro-competent *E. coli* using salt-free growth medium, BioTechniques 20 (1996) 42–44.
[28] P. Carter, H. Bedouelle, G. Winter, Improved oligonucleotide site-directed mutagenesis using M13 vectors, Nucl. Acids Res. 13 (1985) 4431–4443.
[29] M.I. Ramos-González, S. Molin, Cloning, sequencing, and phenotypic characterization of the *rpoS* gene from *Pseudomonas putida* KT2440, J. Bacteriol. 180 (1998) 3421–3431.
[30] V. de Lorenzo, M. Herrero, U. Jakubzik, K.N. Timmis, Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria, J. Bacteriol. 172 (1990) 6568–6572.
[31] H. Pavel, M. Forsman, V. Shingler, An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of *Pseudomonas* sp. strain CF600 on para-substituted methylphenols, J. Bacteriol. 176 (1994) 7550–7557.
[32] M. Herrero, V. de Lorenzo, K.N. Timmis, Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria, J. Bacteriol. 172 (1990) 6557–6567.
[33] S.A. Bayley, C.J. Duggleby, M.J. Worsey, P.A. Williams, K.G. Hardy, P. Broda, Two modes of loss of the TOL function from *Pseudomonas putida* mt-2, Mol. Gen. Genet. 154 (1977) 203–204.
[34] D.H. Figurski, D.R. Helinski, Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 1648–1652.
[35] R. Hõrak, H. Ilves, P. Pruunsild, M. Kuljus, M. Kivisaar, The ColR-ColS two-component signal transduction system is involved in regulation of Tn4652 transposition in *Pseudomonas putida* under starvation conditions, Mol. Microbiol. 54 (2004) 795–807.
[36] L. Kasak, R. Hõrak, M. Kivisaar, Promoter-creating mutations in *Pseudomonas putida*: a model system for the study of mutation in starving bacteria, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 3134–3139.
[37] R.P. Haugland, Assays for cells viability, proliferation and function, in: M.T.Z. Spence (Ed.), Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes Inc., Eugene, 1996, pp. 365–398.
[38] J. Wagner, T. Nohmi, *Escherichia coli* DNA polymerase IV mutator activity: genetic requirements and mutational specificity, J. Bacteriol. 182 (2000) 4587–4595.
[39] R.M. Schaaper, Base selection, proofreading, and mismatch repair during DNA replication in *Escherichia coli*, J. Biol. Chem. 268 (1993) 23762–23765.
[40] E.C. Friedberg, R. Wagner, M. Radman, Specialized DNA polymerases, cellular survival, and the genesis of mutations, Science 296 (2002) 1627–1630.
[41] M.D. Sutton, G.C. Walker, Managing DNA polymerases: coordinating DNA replication, DNA repair, and DNA recombination, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 8342–8349.
[42] F.J. López de Saro, M. O'Donnell, Interaction of the β sliding clamp with MutS, ligase, and DNA polymerase I, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 8376–8380.
[43] F.J. López de Saro, R.E. Georgescu, M.F. Goodman, M. O'Donnell, Competitive processivity-clamp usage by DNA polymerases during DNA replication and repair, EMBO J. 22 (2003) 6408–6418.
[44] B. Dalrymple, K. Kongsuwan, G. Wijffels, N.E. Dixon, P.A. Jennings, A universal protein-protein interaction motif in the eubacterial DNA replication and repair systems, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 11627–11632.
[45] H.I.M. Boshoff, M.B. Reed, C.E. Barry III, V. Mizrahi, DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in *Mycobacterium tuberculosis*, Cell 113 (2003) 183–193.
[46] R.S. Galhardo, R.P. Rocha, M.V. Marques, C.F.M. Menck, An SOS-regulated operon involved in damage-inducible mutagenesis in *Caulobacter crescentus*, Nucl. Acids Res. 33 (2005) 2603–2614.
[47] M. Abella, I. Erill, M. Jara, G. Mazón, S. Campoy, J. Barbé, Widespread distribution of a *lexA*-regulated DNA damage-inducible multiple gene cassette in the Proteobacteria phylum, Mol. Microbiol. 54 (2004) 212–222.
[48] B.G. Hall, Spontaneous point mutations that occur more often when they are advantageous than when they are neutral, Genetics 126 (1990) 5–16.
[49] B.A. Bridges, Hypermutation under stress, Nature 387 (1997) 557–558.
[50] P.L. Foster, Nonadaptive mutations occur on the F'episome during adaptive mutation conditions in *Escherichia coli*, J. Bacteriol. 179 (1997) 1550–1554.
[51] J. Torkelson, R.S. Harris, M.-J. Lombardo, J. Nagendran, C. Thulin, S.M. Rosenberg, Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation, EMBO J. 16 (1997) 3303–3311.
[52] S.E. Finkel, E.R. Zinser, R. Kolter, Long-term survival and evolution in the stationary phase, in: G. Storz, R. Hengge-Aronis (Eds.), Bacterial Stress Responses, American Society for Microbiology, Washington, DC, 2000, pp. 231–238.
[53] J.W. Costerton, Z. Lewandowski, D.E. Caldwell, D.R. Korber, H.M. Lappin-Scott, Microbial biofilms, Annu. Rev. Microbiol. 49 (1995) 711–745.

CURRICULUM VITAE

Signe Saumaa

Date of birth:	28. 01. 1978, Tartu
Nationality:	Estonian
Adress at work:	Institute of Molecular and Cell Biology, University of Tartu 23 Riia Street, 51010, Tartu, Estonia
Phone:	+372 737 5015
email:	siky@ebc.ee

Education and professional employment

1985–1996	Seconday School No.8, Tartu, Estonia
1996–2000	University of Tartu, Institute of Molecular and Cell Biology, BSc in Molecular and Cell Biology
2000–2002	University of Tartu, Institute of Molecular and Cell Biology, MSc in Genetics
2002–present	University of Tartu, Institute of Molecular and Cell Biology, PhD student at Department of Genetics
2006–present	University of Tartu, Institute of Molecular and Cell Biology, research scientist

Scientific work

I have been interested in stationary phase mutational processes in bacterium *Pseudomonas putida* since 1998. My research field has governed the study of mechanisms of stationary phase mutagenesis in *P. putida*, particularly, the involvement of oxidative DNA damage defense systems and DNA mismatch repair pathway in avoidance of stationary phase mutations in *P. putida*.

List of publications

Saumaa, S., Tover, A., Kasak, L. and Kivisaar, M. 2002. Different spectra of stationary-phase mutations in early-arising versus late-arising mutants of *Pseudomonas putida*: involvement of the DNA repair enzyme MutY and the stationary-phase sigma factor RpoS. *J. Bacteriol* **184**: 6957–6965**.**

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CURRICULUM VITAE

Signe Saumaa

Sünniaeg:	28. jaanuar 1978. a., Tartu
Kodakondsus:	Eesti
Aadress tööl	Tartu Ülikooli molekulaar- ja rakubioloogia instituut, Riia 23, Tartu 51010, Eesti
Tel.:	737 5015
E-post:	siky@ebc.ee

Haridus ja erialane teenistuskäik

1985–1996	Tartu 8. Keskkool
1996–2000	Tartu Ülikool, bioloogia-geograafiateaduskond, BSc molekulaar- ja rakubioloogia erialal
2000–2002	Tartu Ülikooli molekulaar- ja rakubioloogia instituut, MSc (teadus) geneetika erialal
2002–…	Tartu Ülikooli molekulaar- ja rakubioloogia instituut, geneetika õppetool, doktorantuuris
2006–…	Tartu Ülikooli molekulaar- ja rakubioloogia instituut, geneetika õppetool, geneetika teadur

Teaduslik töö

Minu teadustöö teemaks on olnud alates 1998 aastast statsionaarse faasi mutatsiooniprotsesside uurimine bakteris *Pseudomonas putida*. Põhiliselt osalen töös, mille eesmärgiks on uurida DNA paardumisvigade reparatsiooni ning oksüdatiivsete DNA kahjustuste eest kaitsvate süsteemide osalust statsionaarse faasi mutatsioonide ärahoidmisel bakteris *P. putida*.

Publikatsioonide nimekiri

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