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Mutagenic potential of DNA damage repair and tolerance mechanisms under starvation stress

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LIST OF ORIGINAL PUBLICATIONS

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

I **Tark M, Tover A, Koorits L, Tegova R, Kivisaar M. (2008)** Dual role of NER in mutagenesis in *Pseudomonas putida*. DNA Repair 7:20–30.

II **Koorits, L., Tegova, R., Tark, M., Tarassova, K., Tover, A., Kivisaar, M. (2007)** Study of involvement of ImuB and DnaE2 in stationary-phase mutagenesis in *Pseudomonas putida*. DNA Repair 6:863–8.

III **Saumaa S, Tover A, Tark M, Tegova R, Kivisaar M. (2007)** Oxidative DNA damage defense systems in avoidance of stationary-phase mutagenesis in *Pseudomonas putida*. J Bacteriol. 189:5504–14.

IV **Tark M., Tover A., Tarassova K., Tegova R., Kivi G., Hõrak R. and Kivisaar M. (2005)** TOL plasmid pWW0-encoded DNA polymerase V homologue confers evolutionary fitness on *Pseudomonas putida* under conditions of environmental stress J. Bacteriol. 187:5203–13.

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

Ref. I designed and performed the experiments, contributed to writing of the manuscript

Ref. II collected data and carried out statistical analyses to validate the data presented in the publication, modeled *P. putida* ImuB catalytic center using *S. solfataricus* DNA polymerase IV Dpo4 as a template, contributed to writing of the manuscript.

Ref. III participated in construction of plasmids and strains, contributed to writing of the manuscript

Ref. IV designed and performed the experiments, contributed to writing of the manuscript

ABBREVIATIONS

6-4PP	pyrimidin-pyrimidone (6-4) photoproduct
8-HDF	8-hydroxy-5-deazoriboflavin
AAF	N-2-acetylaminofluorene
ADP	adenosine diphosphate
AFM	atomic force microscopy
AP site	apurinic/apyrimidinic site; abasic site
ATP	adenosine triphosphate
BER	base excision repair
bp	base pairs
cAMP	cyclic-adenosine monophosphate
CFU	colony forming unit
CPD	cyclobutane pyrimide dimer
DSB	double-strand break
DSBR	double-strand break repair
dsDNA	double-strand DNA
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FRET	fluorescence resonance energy transfer
GASP	growth advantage in stationary phase
GFP	green fluorescent protein
GG-NER	global genomic nucleotide excision repair
GO	7,8-dihydro-8-oxoguanine, 8-oxo-G
HhH	helix-hairpin-helix
Lac	lactose
MAC	mutagenesis in ageing colonies
MMR	mismatch repair
MTHF	5,10-methenyltetrahydrofolate
NER	nucleotide-excision repair
nt	nucleotide
OD	optical density
Phe	phenol
Pol	polymerase
Rif	rifampicin
RLU	relative light unit
RNAP	RNA polymerase
ROS	reactive oxygen species
ROSE	resting organisms in a structured environment
Sm	streptomycin
ssDNA	single-strand DNA
TC-NER	transcription-coupled nucleotide excision repair
TRCF	transcription-repair coupling factor
TLS	translesion DNA synthesis
UV	ultraviolet

INTRODUCTION

There are many endogenous and exogenous sources of DNA damage. The alterations in the chemical structure of DNA that result from damage have potentially tremendous cellular consequences. This is primarily due to interference with processes occurring along DNA, such as transcription and replication.

Cells have developed different means to deal with DNA damage. Lesions can be repaired using different partially redundant systems or the damage in DNA can be tolerated by recruiting specialized DNA polymerases to replicate across the damage. If all attempts to deal with the damage fail, the cell dies. Generally for the individual cell or organism, repair of the damage is preferred over the potentially mutagenic replication carried out by specialized DNA polymerases due to its error-free nature.

On the other hand, there are conditions under which occurrence of mutations is beneficial. Mutations provide the necessary variation for selection and creation of biological diversity. There is evidence from bacteria that the frequency of mutations is a regulated process. For instance in bacteria under conditions of starvation the mutation rate is elevated due to malfunctioning of post-replicative DNA repair and an increase in DNA synthesis carried out by specialized DNA polymerases.

In this thesis I describe the influence of mechanisms of DNA repair and tolerance on mutational processes in bacteria under conditions of carbon starvation. I have focused on the pathways dealing with UV-induced DNA damage. Although the mutagenic potential of these systems is well established under conditions of induced DNA damage, no data is available on the effect of harboring these systems in stationary-phase populations under conditions where no exogenous damage is induced, but endogenous damage is abundant. As a model system we use *Pseudomonas putida*, an ubiquitous soil bacterium. *P. putida* belongs to the genus *Pseudomonas*, one of the most prominent groups of bacteria able to colonize various ecological niches including soil, plants and animals. As pseudomonads live in a constantly changing environment, studying mutational processes in these species provides insights into the processes that make these bacteria fit in adapting to new growth conditions. In this respect *P. putida* has clear benefits as a model organism compared to other models such as *E. coli*, which have much more limited ecological distribution, yet are often used to investigate adaptive processes.

REVIEW OF LITERATURE

Introduction

Heritable information carried in the form of DNA is crucial for cellular integrity. Faithful replication of DNA and its passing on to daughter cells is therefore important for uni- as well as multicellular organisms. Although DNA is a rather stable molecule, it is not inert and it is susceptible to various kinds of chemical modifications. Modifications in DNA (DNA lesions) can occur spontaneously or can be produced either by cellular metabolism itself or by exogenous factors. The group of endogenous agents comprises reactive oxygen species (ROS) and other reactive agents produced in metabolic reactions such as lipid peroxidation. Exogenous damaging agents are numerous; they can be divided into electromagnetic radiation and chemicals. It has been estimated that a mammalian cell is confronted with a spontaneous loss of 750 nucleobases, 4-20 spontaneously deaminated cytosines, 100–1000 oxidative lesions and approximately 50 double-strand breaks in one hour (Vilenchik & Knudson, 2003; Friedberg *et al*, 2006). In addition, exposure to sunlight can cause 30 000 DNA lesions per cell per hour. If left unrepaired, lesions in DNA can lead to cell death by blocking vital processes like replication and transcription or cause mutations if the lesion is bypassed by specialized DNA polymerases. Such polymerases do not remove the lesion, but allow cells to continue functioning in the presence of lesions. Mechanisms that do not lead to removal of damage, but allow the cell to carry out its functions are in general referred to as damage tolerance mechanisms. Also, the repair procedure is not always absolutely exact and error-free. Often DNA repair involves removal of the lesion in a short fragment of the damaged strand and a copying of the intact complimentary strand. Given that the error rates during DNA synthesis for replicative polymerases are between 10^{-6} and 10^{-8}, mutations can be generated as well as fixed during repair synthesis.

While high fidelity DNA synthesis and error-free repair are beneficial for maintaining genetic information over many generations, low fidelity DNA synthesis and emergence of mutations is beneficial for the evolution of species, for generating diversity leading to increased survival of viruses and microbes when subjected to changing environments, and also for mammalians in developing a normal immune system. For instance, in bacteria genetic variability increases in non-favorable growth conditions under which overall chromosome replication is diminished (Tsui *et al*, 1997; Ilves *et al*, 2001; Bjedov *et al*, 2003). Under stress conditions replication fidelity is reduced due to the involvement of specialized DNA polymerases and absence of repair of replication errors. Also, chromosome rearrangements occur with higher frequency and some transposable elements are induced. This increase in mutational processes is not random, but regulated through several pathways such as the SOS-response and the general stress response.

The literature review section of my thesis consists of two separate parts. In the first part I will give an overview of responses of bacteria to DNA damage and describe the repair systems involved in removal of exogenous DNA damage induced by exposure to sunlight as well as the damage tolerance mechanisms involved. In the second part I will describe bacterial life in natural conditions and give an overview of processes occurring in bacteria under conditions of stress.

I MECHANISMS TO REPAIR AND TOLERATE UV-INDUCED DNA DAMAGE IN BACTERIA

Studies on the repair of DNA damage upon exposure to UV-light or ionic radiation, as well as on the resulting mutagenic phenotype laid the basis for the field of DNA repair and mutagenesis in bacteria (Friedberg *et al*, 2006). Half a century after these pioneering studies, we have a fairly extensive understanding not only of these processes, but we also know what are the events occurring in response to encountering DNA damage. In this part of my thesis I give an overview of what is known to date, trying to keep to the sequential order of events as occurring in a cell upon encountering the damage. Thereafter I give an overview of the repair mechanisms available for removal of UV-induced damage and describe the ways in which DNA damage can be tolerated.

1. UV radiation

Ultraviolet (UV) light is electromagnetic radiation with a wavelength shorter than visible light and longer than X-rays, comprising the range of 400-100 nm. According to its wavelength UV light is divided into three distinct spectral areas. These are UV-A (400-320 nm), UV-B (320-295 nm) and UV-C (295-100 nm). UV light is part of the light spectrum emitted by the Sun. Not all of the UV radiation reaches the Earth's surface due to the presence of the stratospheric ozone layer. UV-A radiation and a fraction of UV-B penetrate the ozone layer, while all of the UV-C and most of the UV-B is absorbed within.

UV-induced DNA damage

UV-C and UV-B radiation are absorbed by DNA, lipids and proteins and cause damage to all of them. UV-A is generally considered harmless. As the aim of my thesis is to investigate UV-induced DNA damage and the way bacteria cope with it, I will focus on the effect of UV irradiation on DNA. The damage in DNA in general may cause permanent changes in hereditary information and therefore have harmful effects for the individual. The accumulation of UV-

induced DNA damage in unicellular organisms leads to their death, while in humans for instance, it has been shown to be the main reason for premature ageing and skin cancer (Pfeifer, 1997). Therefore it is important to study the mechanisms by which UV-induced damage arises and how it is dealt with in different organisms. Most of the experiments with bacteria and cell cultures in which UV-induced DNA damage and its effects are studied are done in conditions where damage is induced with UV-C light, with a wavelength of 254 nm. The rationale behind this choice is that (1) UV-C and UV-B cause the same types of DNA damage; (2) UV-C 254 nm is a relatively specific DNA damage inducer as this wavelength is close to the absorption peak of DNA at 260 nm, whereas this wavelength is not absorbed in proteins (Friedberg *et al*, 2006).

UV-B and UV-C damage nitrogenous bases in DNA. This damage is caused by covalent bonding of adjacent pyrimidine bases (C, T or U). Dimers between adjacent pyrimidines may form in two distinct ways. The most frequent UV-induced DNA lesions (approximately 75%), are cyclobutane–pyrimidine dimers (CPDs) in which a cyclobutane ring connects the C5 and C6 positions of both adjacent pyrimidines (Figure 1A). In 25% of the lesions a single covalent bond has formed between the C6 position of one pyrimidine and the C4 position of the adjacent pyrimidine on the 3´side giving rise to a so called 6-4 photoproduct (6-4PP) (Figure 1A). Most commonly dimers are formed in a thymine pair or between adjacent thymine and cytosine (Friedberg *et al*, 2006). Purines (A, G) are generally considered to be resistant to UV damage. Nevertheless, photo-products involving a purine and an adjacent pyrimidine have also been reported (Sinha & Häder, 2002). In addition to CPDs and 6-4PPs, UV irradiation also induces, albeit at a much lower frequency, pyrimidine monoadducts. Photo-addition of H- or HO- to the double bonds at positions C5 and C6 of thymine and cysteine results in formation of thymine glycol and cytosine hydrate, respectively (Figures 1B and 1C). Cytosine hydrates are further converted to uracil hydrates (by loss of the amino group) and after subsequent dehydration yield uracil in DNA (Figure 1C) (Boorstein *et al*, 1990). Pyrimidine mono-adducts are thought to arise in DNA at a frequency of only 1–2% of that of CPDs and therefore have been much less studied than CPDs and 6-4PPs (Mitchell *et al*, 1991).

CPDs and 6-4PPs create distortions in DNA, bending it by 7-9° and 44°, respectively. Due to this bending and the loss of normal base-pairing these lesions are obstacles to cellular processes such as DNA replication and transcription (Kim *et al*, 1995). It is no surprise that due to the major distortion induced in DNA, 6-4PPs have more serious, potentially lethal and mutagenic effects, while the toxicity of CPDs is limited and mostly due to the abundance of this type of the damage (Sinha & Häder, 2002). Although a single lesion if not removed is sufficient to block transcription and replication, only a small fraction of CPDs and 6-4PPs results in mutations in the original and replicated DNA. Both dimers can react further under certain conditions and form more mutagenic compounds than CPDs and 6-4PPs themselves (Horsfall & Lawrence, 1994). On continuous exposure to wavelengths longer than 290 nm, most

of the 6-4PPs are converted to Dewar valence isomers (Taylor *et al*, 1990). DNA replication across a T-C Dewar isomer has been shown to be more erroneous than across T-C 6-4PP. Cytosines in CPDs can 'spontaneously' loose the amino group and as a result be converted into uracil. As cytosine pairs to guanine but uracil to adenine, such deamination gives rise to C→T mutations (Barak *et al*, 1995).

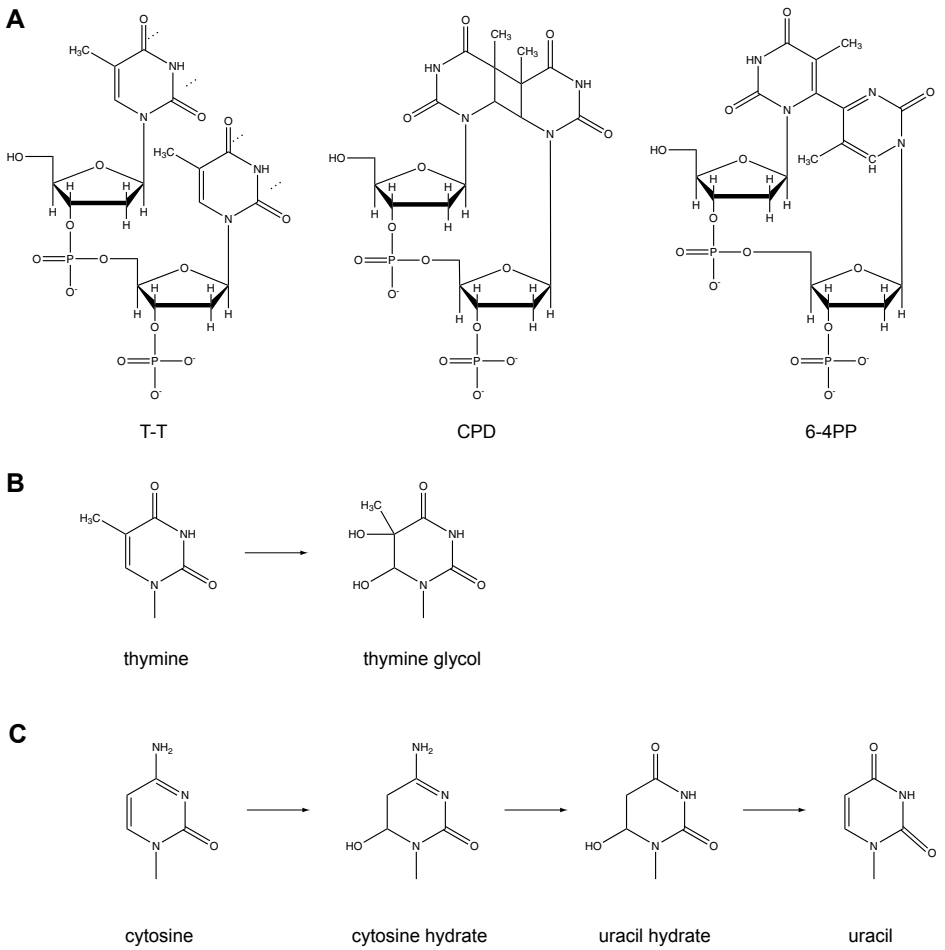


Figure 1. UV-induced DNA damage. **A.** Structures of two undamaged adjacent pyrimidines (thymidines,T-T), a cyclobutane-pyrimidine dimer (CPD) and a 6-4 photoproduct (6-4PP). The sugar-phosphate backbone is shown to illustrate the distortion induced by dimer formation. In the case of the undamaged thymidine dimer, T-T, hydrogen bonding with complimentary bases is indicated by dotted lines. **B.** Formation of thymine glycol. **C.** Formation of cytosine hydrate and its further conversion to uracil hydrate and uracil.

Induction of UV damage in DNA is not random, but is determined by the sequence and structure of the DNA. As both CPDs and 6-4PPs affect pyrimidines, the susceptibility of DNA is determined by the occurrence of this type of nucleobases in the DNA sequence. The formation of CPDs and 6-4PPs needs the DNA to bend; therefore this type of damage occurs most frequently at flexible DNA regions at the ends of poly (dA)-(dT) tracts and in melted, single-stranded DNA regions (Becker & Wang, 1989).

UV-A radiation is *per se* less efficient in inducing DNA damage than UV-B and UV-C radiations as it is not absorbed by DNA. However, it can still lead to secondary photoreactions of existing DNA photoproducts (e.g. formation of Dewar valence isomers) or damage DNA by indirect photosensitizing reactions (Wei *et al*, 1998). As UV-A causes indirect damage similar to that produced by reactive oxygen species, these types of lesions and their effects on cellular processes are described in this thesis in the section focused on endogenous DNA damage, "Endogenous oxidative damage in starving bacteria". Although UV-A has been generally considered a harmless fraction of UV radiation, recent findings challenge that belief and show that UV-A can also produce CPDs in DNA (Jiang *et al*, 2009b).

2. Response to UV-induced DNA damage in bacteria

How bacteria react to DNA damage depends on several factors, but most important is the nature and abundance of the encountered damage. CPDs and 6-4PPs induced by UV irradiation have been shown *in vitro* to be insuperable lesions for the main DNA polymerases participating in chromosome replication in *E. coli*, DNA pol III and DNA pol I (Moore *et al*, 1981). However, *in vivo,* exposure to UV irradiation does not always lead to replication arrest. The events following DNA damage induction in a bacterium are summarized in this section.

2.1. Cellular processes upon encountering DNA damage

E. coli growing in a nutrient rich environment has a doubling time of 30–40 minutes. It has been shown that the bacterium is capable of copying its genome of 4.6 Mbp from a single replication origin with high fidelity in less than 40 min. The replication machinery has evolved to keep the replication fork moving as quickly as possible even though it is constantly challenged by various types of DNA damage. DNA replication is carried out in bidirectional fashion by the DNA polymerase III holoenzyme that contains two or three copies of the pol III core, the processivity factor β sliding clamp and the γ/τ clamp loader complex (Figure 2) (O'Donnell, 2006; McInerney *et al*, 2007). Attachment of the DNA pol III cores to the same clamp loader guarantees simultaneous synthesis of the leading and the lagging DNA strands.

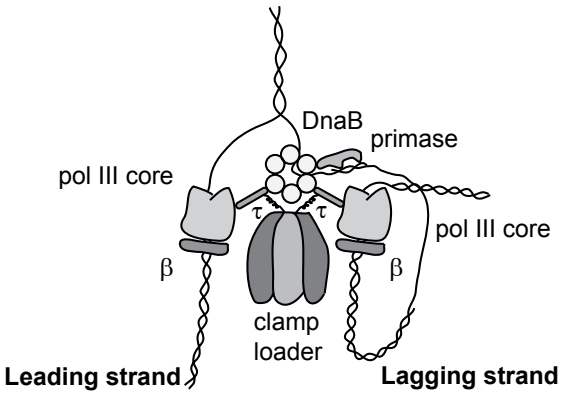


Figure 2. Architecture of the *E. coli* replication fork. The parental duplex DNA is unwound by the DNA helicase DnaB. DnaB encircles the lagging strand ssDNA and primase (DnaG) interacts with DnaB for the synthesis of an RNA primer on the lagging strand. The polymerase III holoenzyme is composed of two pol III cores, one on the lagging strand and one on the leading strand. The pol III cores are held to their respective DNA strands by the β-clamp, which is loaded onto primed sites by the clamp loader complex. The complex consists of one δ, one δ', one γ and two τ subunits; the C-terminal extensions of τ bind pol III core and DnaB. A third DNA pol III core (not shown) may be used to allow rapid reinitiation of replication on either of the DNA strands upon encountering replication-blocking DNA damage (Yao & O'Donnell, 2008). Figure adapted from Leu *et al* (2003).

Based on *in vitro* experiments on T7 and T4 phage replisomes it was initially suggested that synthesis of the two DNA strands is tightly coupled. Blocking progression by one pol III core due to the presence of a DNA damage was also thought to halt the polymerase on the other strand (Lee *et al*, 1998; Salinas & Benkovic, 2000). Later studies have revealed that whether the replication is stopped on both DNA strands or not depends on which of the two strands the blocking lesion was encountered. Stalling of the polymerase on the lagging strand does not stop nor change the rate of replication carried out by the polymerase acting on the leading strand (Higuchi *et al*, 2003; Pagès & Fuchs, 2003; McInerney & O'Donnell, 2004). Instead, leading and lagging strand synthesis becomes uncoupled even though the two polymerases remain connected via the γ/τ clamp loader complex. The stalled DNA polymerase is thereafter prematurely released from its β-clamp at the location of the damage and replication of the lagging strand is resumed downstream the lesion (Wang, 2005). The resulting single-stranded DNA (ssDNA) gap(s) containing a lesion is/are thereafter repaired *via* RecA-mediated recombinational repair (described in the section "Tolerance to UV-induced DNA damage: Single-strand DNA gap-filling". Although there is some evidence that leading-strand synthesis can

also be re-initiated downstream the DNA damage (as described for the lagging stand) (Heller & Marians, 2006; McInerney *et al*, 2007), earlier observations suggest that the damage encountered on the leading strand is usually believed to result in a delay of replication for a minimum of 15–20 minutes before resuming at the original rate (Higuchi *et al*, 2003; Pagès & Fuchs, 2003). As replication of the undamaged lagging strand still continues for a short time, the leading strand template becomes exposed (Higuchi *et al*, 2003; Pagès & Fuchs, 2003; McInerney & O'Donnell, 2004). The exposed strand is covered by RecA proteins, forming a nucleoprotein filament that acts both to induce the SOS repair response and to provide means to process and rescue the damaged fork. Replication restarts after the lesions have been removed and an entirely new replisome is assembled. Prior to the restart of a blocked replication fork several recombinational events occur. These mechanisms will not be discussed in this thesis. Observations that replication restart is tremendously delayed in UV irradiated nucleotide excision repair deficient cells, suggest that replication restart and lesion removal might be coupled (Rudolph *et al*, 2007). According to the model that lesions are skipped and replication is re-initiated on the leading strand as well as on the lagging strand, the ssDNA gaps left behind serve as a signals triggering SOS response (Yao & O'Donnell, 2008).

DNA replication is not the only process on DNA that is hampered by UV-induced DNA damage. Transcription by RNA polymerase (RNAP) is also blocked when confronted with a DNA lesion. It has been shown that stalled RNAP is an insuperable block for DNA replication and needs to be removed for replication and transcription to continue (McGlynn & Lloyd, 2000). RNAPs stalled at a lesion are recognized by transcription repair coupling factor Mfd (Selby & Sancar, 1993). The protein binds to stalled RNAP and induces with its C-terminal dsDNA translocase domain forward movement (in the direction of RNA synthesis) of RNAP to resume the transcription. If transcription does not resume promptly, Mfd induces dissociation of RNAP from its transcript and the DNA (Park *et al*, 2002). Thereafter, Mfd still bound to the DNA recruits NER by binding to UvrA *via* its N-terminal domain (Selby & Sancar, 1993; Assenmacher *et al*, 2006). Mfd recognizes and dissociates RNAPs that have stalled for any reason, and thus the protein is not specific for certain types of DNA damage. NER that follows the recruitment to the lesion by Mfd is called transcription-coupled NER (TC-NER). A detailed overview of NER and TC-NER can be found in this thesis in the section “Repair of UV-induced DNA damage: Nucleotide excision repair”.

2.2. Signals produced in response to DNA damage SOS response in *E. coli*

Upon severe DNA damage, single-stranded DNA gaps become abundant in the cell. These ssDNA regions are covered by RecA proteins to form a RecA nucleoprotein filament. The RecA nucleoprotein filament (RecA*) has two

functions; it may either catalyze the strand exchange reaction in homologous recombination, or induce the DNA damage response (SOS response) (Kuzminov, 1999). If RecA* is formed and persists, the SOS signal is given to the cell and genes in the so called 'SOS regulon' are expressed. This regulon consists of more than 40 unlinked genes the products of which are involved in cell cycle regulation, DNA repair and DNA damage tolerance. The regulon is under the control of transcriptional repressor LexA. *lexA* belongs to the SOS regulon and its transcription is therefore autoregulated. Under favourable growth conditions LexA is bound to its binding site (SOS box, LexA box) overlapping the promoter/operator area of each gene in the SOS regulon, preventing RNA polymerase binding and gene expression in this way. Yet, some SOS genes, including *lexA* itself and *recA*, are also transcribed in uninduced cells. The SOS box has a palindromic sequence with the consensus 5′-CTG$(N)_{10}$CAG-3′ in *E. coli* and LexA binds to it as a dimer (Thliveris *et al*, 1991; Jara *et al*, 2003). Although generally only one SOS box is present in one gene or operon, there are some exceptions: *lexA* and a *ydjM* gene with an unknown function contain two adjacent SOS boxes and *recN* even three (Koch *et al*, 2000). All of these boxes are functional and bind LexA in a cooperative manner. In the presence of RecA* the autocatalytic activity of LexA is activated and the protein undergoes a selfcleavage reaction (Figure 3). Within few minutes after damage induction the LexA pools decrease nearly tenfold, albeit its constant resynthesis (Michel, 2005). This decrease in levels of active LexA vacates the promoter areas of genes in the SOS regulon and allows their transcription.

Not all SOS regulon genes are induced at the same moment. Temporal activation of genes is achieved by differential affinity of LexA to its binding sequences. The number of SOS boxes in a gene does not show any correlation with its induction time (Koch *et al*, 2000). Genes expressed most rapidly after DNA damage include the transcriptional repressor of the operon, LexA and proteins participating in nucleotide excision repair (NER) UvrA, UvrB, Cho and UvrD. Immediate expression of *lexA* guarantees, that the repression of the SOS regulon is re-established as soon as the damage signal RecA* disappears. In the next stage the expression of genes encoding the recombinational DNA repair proteins RuvA, RuvB, RecN and RecA, specialized DNA polymerases DNA pol II and pol IV and a protein affecting RecA* coprotease activity, DinI, is enhanced. Genes encoding the cell division inhibitor *sulA* and *umuDC* are derepressed at the latest stage of SOS induction (Courcelle *et al*, 2001). *umuDC* products have two distinct, temporally separated roles after damage induction. UmuD can undergo RecA* mediated proteolytic cleavage and therefore exists in two forms: the unprocessed form UmuD and the processed form UmuD′. UmuC in complex with UmuD′ (UmuD′$_2$C) has DNA polymerase activity and is referred to as DNA pol V (Tang *et al*, 1999). The role of UmuD′$_2$C is discussed in detail in the section "Tolerance to UV-induced DNA damage: *E. coli* DNA polymerase V". UmuC in complex with UmuD (UmuD$_2$C) has no DNA polymerase activity. This complex acts as a primitive DNA damage

checkpoint by blocking DNA replication directly and allowing additional time for DNA repair to act (Opperman *et al*, 1999). Proteolysis of UmuD is delayed by DinI due to its competitive binding to RecA* thus inhibiting its coprotease activity (Yasuda *et al*, 2001). It has been shown that after UV irradiation, uncleaved UmuD predominates over UmuD′ for 20 minutes, after which UmuD′ becomes the predominant form (Opperman *et al*, 1999). After UmuD′ accumulation, UmuD′$_2$C complexes are formed.

Using different reporter systems and methods to measure the promoter activity of SOS genes it has been shown that transcription of SOS genes increases after induction of DNA damage (UV irradiation) and ceases abruptly after 45–60 minutes. Also, the recovery of DNA synthesis is observed approximately 45 minutes after exposure to UV (Friedberg *et al*, 2006). During these 45–60 minutes of SOS induction, cell division is inhibited and the damage is actively repaired using various mechanisms. After successful DNA repair, single-stranded DNA regions have been removed, and the cellular damage signal RecA* cannot form anymore (Figure 3). In the absence of RecA*, the repressor protein of the SOS regulon, LexA, cannot be cleaved. As the intact LexA pools increase, the repression of the SOS regulon genes is regained and cells can continue their normal growth. However, not all bacterial cells survive exposure to damage. Those that have accumulated lesions that cannot be repaired or that have extreme amounts of damage die. For instance, on exposure to UV radiation at a dose of 40 J/m^2 about half of *E. coli* cells fails to resume growth on a nutrient plate (Thoms & Wackernagel, 1988).

Experiments measuring the SOS response in individual cells using green fluorescent protein (GFP) as a reporter for promoter activity have shed more light on the nature of SOS induction. It has been generally assumed that single induction of SOS genes occurs after encountering DNA damage. Measurements of the promoter activity of three SOS genes, *recA*, *lexA* and *umuDC*, on the contrary showed several peaks of SOS induction over a period of 120 minutes after bacteria were exposed to UV (Friedman *et al*, 2005). The number of SOS induction peaks observed increased with the amount of damage. The expression level and duration of each single induction peak was less affected. It suggests that cells respond to increased damage levels by increasing the number of SOS induction cycles, rather than by increasing the time of the response. The observed behavior of SOS induction cannot be explained simply by transcriptional control by LexA, but suggests the existence of a more complex level of regulation.

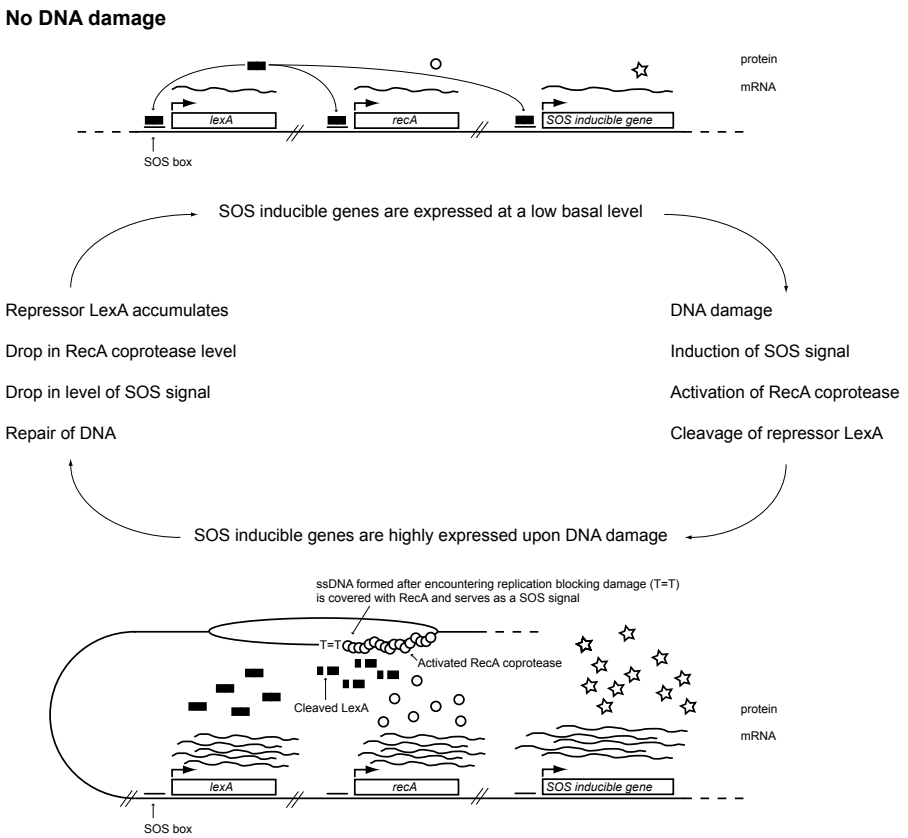


Figure 3. The SOS response in *E. coli*. When no damage is encountered LexA is bound to promoters of SOS inducible genes and the genes are expressed at a low basal level. After encountering replication blocking DNA damage (T=T) ssDNA is formed and subsequently covered with RecA. The RecA nucleoprotein filament (RecA*) serves as SOS signal. RecA* mediates autocleavage of LexA, LexA pools decrease and the protein frees the promoter areas of SOS inducible genes resulting in increased expression levels. After DNA damage is repaired, ssDNA is not available and RecA* cannot form. As RecA* is not there to mediate LexA autocleavage the repressor accumulates and binds to its binding sites at the promoters of SOS inducible genes. Figure adapted from Friedberg *et al* (2006).

SOS response in other bacteria

SOS response is a universal adaptation response of bacteria to DNA damage. Systems similar to that characterized in *E. coli* have been found in many other bacterial species. Most commonly, the genes belonging to the SOS regulon function in recombinational repair, excision repair and encode DNA

polymerases capable of translesion synthesis. Based on the fact that all these mechanisms contribute to UV resistance, it has been hypothesized that the aim of the SOS response is mainly to overcome the DNA damage induced by UV radiation. In most bacterial species, *recA* and *lexA*, the positive and negative regulators of the response, respectively, are also part of the SOS inducible regulon.

The recognition sequence of the LexA proteins varies drastically by species (Erill *et al*, 2003; Erill *et al*, 2007). Also the number of genes as well as the genes that are part of the SOS regulon is different. For instance, in *E. coli* and *Bacillus subtilis*, the SOS regulon includes a relatively large number of genes: at least 43 and 33, respectively, but only eight of these genes are shared between the two species (Courcelle *et al*, 2001; Au *et al*, 2005). In contrast, the regulons of the more pathogenic bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, contain only 16 and 15 genes, respectively (Cirz *et al*, 2006b; Cirz *et al*, 2007). Genes that have escaped negative regulation by LexA, are either constitutively expressed or regulated in other ways.

Several *Pseudomonas* and *Xanthomonas* species possess two *lexA* genes, the products of which recognize different LexA binding sequences. Genomic microarray analysis of the *Pseudomonas putida* expression profile indicates that one of the two LexA proteins (LexA1) is under the control of the conventional *E. coli*-like SOS response, while the other LexA (LexA2) regulates only its own transcriptional unit, which includes the *imuA, imuB*, and *dnaE2* genes, and a gene from a resident *P. putida* prophage (Abella *et al*, 2007). The genes encoded in the operon and their functions are described in the section on "Tolerance to UV-induced DNA damage: DnaE2 proteins associated with UV-mutagenesis". The LexA2 operon is likely of acinetobacterial origin, and is widely dispersed across the bacterial domain through a combination of vertical inheritance, lateral gene transfer and duplication (Erill *et al*, 2007). *In silico* analyses have revealed that in genomes of several bacteria, *P. aeruginosa*, *Vibrio parahaemolyticus* and *Shewanella oneidensis,* the *imuA-imuB-dnaE2* cassette does not encode the second LexA homologue. Instead, the binding sequence of LexA1 is found in the promoter area of these genes, hence suggesting their regulation by LexA1 (Erill *et al*, 2006b).

There are several bacteria, mostly extremophiles, *Dehalococcoides ethenogenes*, *Bdellovibrio bacteriovorus*, *Magnetococcus spp.*, *Petrotoga miotherma*, and *Geobacter sulfurreducens*, in which the so far identified network of SOS inducible genes consists of only one or a couple of transcription units (Fernández de Henestrosa *et al*, 2002; Jara *et al*, 2003; Campoy *et al*, 2005; Erill *et al*, 2006a). In these bacteria as well as in *Deinococcus radiodurans* (Narumi *et al*, 2001), expression of *recA* is LexA independent. In *Leptospira interrogans*, LexA is not subject to autoregulation and in *Thermotoga maritima* neither RecA nor LexA is part of the SOS regulon (Cune *et al*, 2005; Erill *et al*, 2006a).

In spite of the universality of the SOS response, there are several bacteria that do not encode LexA homologues. No *lexA* sequence homologues have been

detected in *Helicobacter pylori*, *Campylobacter jejuni*, *Rickettsia prowazekii*, *Buchnera aphidicola*, *Chlamydia trachomatis*, *Borrelia burgdorferi*, *Mycoplasma pulmonis*, *Neisseria gonorrhoeae*, *Thermus thermophilus*, *Aquifex pyrophilus*, *Bacteroides fragilis, Streptococcus spp.* and *Lactococcus spp.* (Cirz *et al*, 2006a; Erill *et al*, 2007). Most of these bacteria are pathogens and have undergone major genomic reduction. Possibly loss of *lexA* and gain of constitutive expression of repair genes is beneficial in their environment. It is also plausible that these bacteria have evolved an SOS response distinct from the LexA-RecA mechanism. In *S. pneumoniae* the SOS genes are controlled as part of the competence regulon. There is also evidence that in *Streptococcus* and *Lactococcus* spp, the damage inducible genes are controlled by the LexA-like transcriptional repressor HdiR (Savijoki *et al*, 2003; Varhimo *et al*, 2007).

3. Repair of UV-induced DNA damage

In a cell that has been exposed to solar light, the total number of nucleotides damaged by UV is very small compared to the size of the entire genome. For instance, UV-B light with a wavelength of 300 nm at an intensity of approximately 230 J/m^2 causes one pyrimidine dimer per 10^3 basepairs (Jiang *et al*, 2007). As the size of bacterial genomes is in general in the order of magnitude of 10^6 basepairs, such a UV dose would result in approximately 1000 pyrimidine dimers per genome. These calculations were done using extracted DNA and therefore the amount of lesions reported exceeds the damage induced into DNA in the intracellular environment. Due to differences in the structure of cell membrane, DNA packing and for instance exopolysaccharides secreted, the radiation that reaches the DNA in a bacterium may vary. As mentioned before, even one pyrimidine dimer if not repaired is capable of blocking replication and transcription, which in turn interferes with all other metabolic processes within the cell. To reduce the threat to viability due to solar UV exposure bacteria have evolved multiple and diverse mechanisms to deal with the damage. In this section of my thesis I will describe molecular mechanisms of the most common repair systems which main function is removal of UV-induced DNA damage.

3.1. Enzymatic photoreactivation

The simplest of the UV-induced DNA damage repair mechanisms is reversal of pyrimidine dimers. This is carried out in a single step reaction in which covalently joined adjacent pyrimidines are reversed to pyrimidine monomers by a photoreactivating enzyme (photolyase) in the presence of visible light in the range 300–500 nm, referred to as enzymatic photoreactivation (described in more detail in Figure 4). Alternatively, the dimers can be reversed directly by the light that is absorbed in photodimers (235 nm), in a process called direct reversal. Reversal of pyrimidine dimers is considered the most accurate and

efficient way to remove damage caused by UV irradiation from DNA because it does not involve removal of damaged nucleotides and resynthesis as in the case of other repair pathways described later in this thesis. Since the wavelengths necessary for direct reversal are the same that cause UV-induced damage, direct reversal is not considered to be a repair of biological significance (Friedberg *et al*, 2006). The benefit for a cell is that at high UV light intensities the formation of pyrimidine dimers and their reversal reaches a steady state: as many dimers as are formed are reversed, yielding a constant amount of lesions in DNA despite the increase in UV dose. The direct reversal process removes only CPDs from DNA. Enzymatically, both CPDs and 6-4PPs can be removed. These lesions are reversed by different enzymes, CPD photolyases and (6-4) photolyases, respectively. CPD photolyases are common in all kingdoms of life, while 6-4 photolyases have only been found in plants to date (Sancar, 2008). CPD and (6-4) photolyase together with cryptochromes belong to the photolyase/cryptochrome protein group, which constitutes a large flavoprotein family (Partch & Sancar, 2005). These enzymes are evolutionarily related but functionally distinct. CPD and (6-4) photolyases use a blue-light photon as a co-substrate for UV-induced damage reversal while cryptochromes use the photon to regulate light related processes as circadian clock in animals and light-dependent growth in plants (Ahmad *et al*, 1998; Panda *et al*, 2002).

As only CPD photolyases are found in bacteria, I will discuss here this type of photolyase in more detail. Sequence comparison has revealed that approximately half of the bacterial species encode homologues of CPD photolyases. Distribution of CPD photolyase homologues among bacteria shows no correlation with their phyllospheric or other potentially sun-exposed habitat (Goosen & Moolenaar, 2008). The best characterized example of bacterial photolyases is the CPD photolyase from *E. coli*, encoded by the *phr* gene. There are about 10–20 molecules of the enzyme per cell in *E. coli* stationary-phase cultures and the expression of photolyase is induced upon exposure to light (Harm *et al*, 1968). In the presence of visible light of suitable wavelength a photolyase molecule can hydrolyze approximately five CPDs per minute *in vivo*, while *in vitro* the efficiency has been shown to be ten times higher (Harm, 1970; Li & Sancar, 1991). This difference in efficiency is considered to be the result of random diffusion of the proteins in the cell, which makes recognizing a new CPD a time-consuming event for the enzyme (Gruskin & Lloyd, 1988). Regardless of the increase in photolyase levels upon exposure to sunlight, the amount of photolyase remains insufficient to remove in *E. coli* CPDs formed by natural sunlight at midday (Harm *et al*, 1968). Therefore, for removal of UV-induced damage assistance of other repair pathways is needed.

E. coli CPD photolyase is a damage-specific enzyme that has a 100 times preference for binding to CPD-containing DNA compared to undamaged DNA (Sancar *et al*, 1985). Although the damage reversal by photolyase is a light-dependent process, binding to the damage does not need light and is therefore known as the ‘dark reaction’ of the pathway. Differently from many other repair proteins that move processively on DNA, i.e. bind to undamaged DNA by

random collision and continue scanning the DNA for damage, photolyase locates the damage by random diffusion, binding to DNA and dissociating from it continuously (Gruskin & Lloyd, 1988). Taking into consideration the low amount of photolyase present in cells, it does not seem to be the most effective way to find the damage.

Not all CPDs are recognized by photolyase with equal efficiency. As the damage recognition properties of this protein rely mostly on the changes that CPDs induce in the DNA backbone, CPDs inducing a larger deviation from normal DNA structure are better recognized. Thus thymine dimers are recognized the best by photolyase, while cytosine dimers are the worst. In addition to cytosine and thymine adducts photolyase binds well to uracil dimers in the context of DNA, but these are badly recognized in RNA (Kim & Sancar, 1991). Binding to the lesion by CPD photolyase has been shown to be the rate limiting step in the repair. After the damage is bound, the reversal rate of different CPDs is equal. CPDs bound by photolyase in the absence of light can be reversed immediately on exposure to light of an appropriate wavelength. However, light-dependent reversal seems not to be the only way of CPD removal by photolyase. Experiments show that the presence of photolyase enhances the activity of nucleotide excision repair (NER) (Sancar *et al*, 1984). This observation suggests that photolyase can serve as an accessory damage recognition factor for NER.

For splitting the cyclobutane ring connecting two adjacent pyrimidines in CPDs photolyase uses energy from visible light. The light energy is captured by two chromophores bound non-covalently to the enzyme: the catalytic cofactor FAD (absorption maximum at 360 nm) and the light-harvesting antenna 5,10-methenyltetrahydrofolate (MTHF) (absorption maximum at 385 nm) (Sancar, 1994). 8-hydroxy-5-deazariboflavin (8-HDF), FMN or FAD is used as the second chromophore in some bacterial species, mostly in thermophilic bacteria (Ueda *et al*, 2005; Fujihashi *et al*, 2007). The second chromophore is not essential for repair, but allows increasing the repair efficiency by absorbing a light photon and transferring it to $FADH^-$, the active form of the catalytic cofactor FAD (Takao *et al*, 1989; Jorns *et al*, 1990). To initiate damage reversal CPD-bound photolyase needs to take up a photon (Figure 4). This photon is absorbed either directly by $FADH^-$ or is transferred to $FADH^-$ from MTHF in the form of electron. Subsequently, flavin that is excited by the energy from the photon ($FADH^{*-}$) transmits an electron to the CPD to induce splitting of the cyclobutane ring. After the CPD is split into monomers, the electron is transferred back to $FADH^{\bullet}$ to restore $FADH^-$ (Sancar, 2008). The $FADH^-$ bound photolyase can thereafter bind the next CPD and catalyze its reversal.

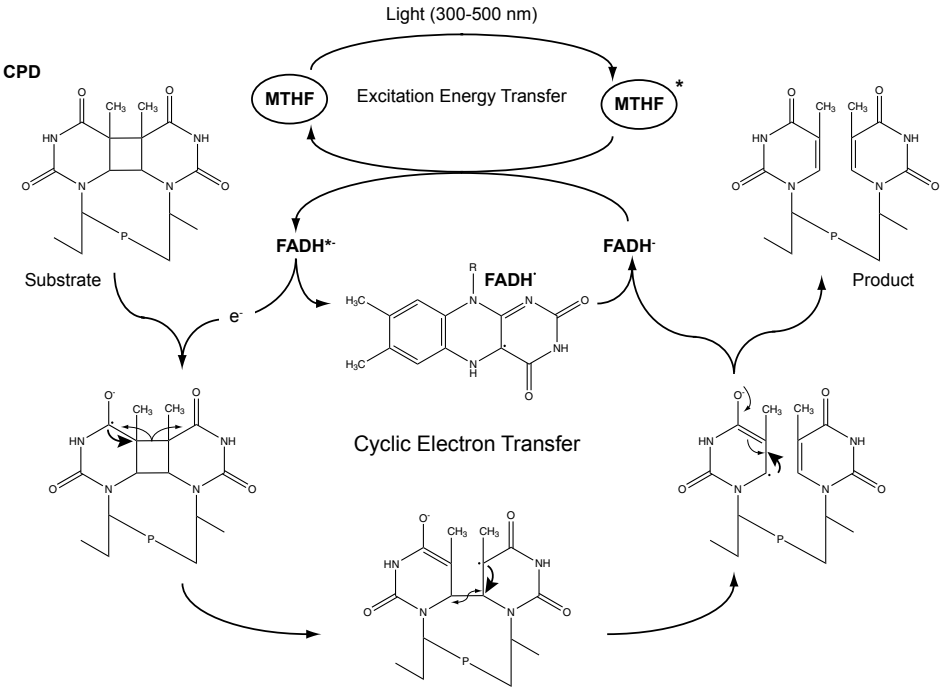


Figure 4. Reaction mechanism of *E. coli* photolyase. A light photon is transferred to $FADH^-$ from MTHF (5,10-methenyltetrahydrofolate) in the form of an electron. $FADH^{*-}$ transmits the electron to CPD. In subsequent steps the cyclobutane ring connecting the two adjacent pyrimidines in the CPD is split and the electron is transferred back to $FADH^{\bullet}$ to restore its initial state, $FADH^-$. Figure adapted from Sancar (2008).

Insights into the mechanism of photolyase function have been obtained from crystal structures of enzymes solved in the presence or absence of DNA with a CPD. Structures have been solved for CPD photolyase from *E. coli*, *Thermus thermophilus* and *Anacystis nidulans*, each of which exhibits similar features that will be discussed below (Park *et al*, 1995; Tamada *et al*, 1997; Komori *et al*, 2001; Mees *et al*, 2004). Photolyases are globular proteins consisting of two domains that are connected through a linker region. The MTHF light harvesting antenna locates in the cleft between the two domains, while FAD is buried deep into the C-terminal domain. A positively charged groove that facilitates contact with the DNA runs across the protein. In the middle of that groove is the active site of the protein, a cavity with the proper dimensions and polarity to accommodate a CPD. Strong interactions occur between the positively charged groove on the photolyase surface and the γ phosphate of the 3´ pyrimidine of the CPD. Additional weak interactions occur with the complimentary DNA strand across the CPD. These interactions weaken the stability of the DNA duplex in the immediate vicinity of the CPD and the CPD is 'flipped out' into the active

site of the photolyase. This structural change results in the formation of a complex of high stability between the CPD and photolyase. Within this complex, the CPD is in van der Waals contact with $FADH^-$ in such a manner that high efficiency electron transfer can occur upon binding of a photon by $FADH^-$ and catalyze monomerization of the CPD.

3.2. Nucleotide excision repair

Nucleotide excision repair (NER) is the most widespread UV-induced damage repair pathway. It is found in most of the prokaryotes and eukaryotes and its mechanism is conserved. The pathway involves recruitment of several proteins acting in sequential manner to excise a lesion from the DNA and resynthesise the resulting gap. In addition to UV-induced damage, NER recognizes and repairs several other types of naturally occurring (oxidative and alkylating damage) and synthetic lesions. Although known by its ability to remove UV-induced DNA damage *in vivo*, it is not clear whether this is the main substrate for this repair system in cells. The importance of NER in the removal of UV-induced lesions is evidenced by the observation that loss of NER function results in severe UV sensitivity; death of microorganisms upon exposure to UV and defects associated with sunlight sensitivity in humans: *Xeroderma pigmentosum*, trichothiodystrophy and Cockayne syndrome.

As mentioned above, NER is a highly conserved pathway. Regardless of the organism, it progresses through the same basic steps: DNA damage recognition, excision of the damage in a short DNA fragment (12–13 nt in prokaryotes and 25–30 nt in eukaryotes) and resynthesis of the DNA (Figure 5A). While in bacteria, NER is carried out by three repair-specific proteins, in eukaryotes at least nine proteins are needed to accomplish the same. NER is divided into two subpathways, global genomic NER (NER, GG-NER) and transcription-coupled NER (TC-NER). These pathways differ only in the damage recognition process. While in GG-NER damage is located by NER-specific proteins, TC-NER makes use of RNA polymerase stalled on DNA upon encountering a lesion during the transcription process (Figure 5B).

Among bacteria, NER has been the best characterized in *E. coli*. The repair is carried out by UvrA, UvrB, UvrC proteins together with the DNA helicase UvrD, DNA pol I and DNA ligase that participate in removal of the DNA fragment containing the lesion and subsequent DNA resynthesis. The NER-specific proteins UvrA and UvrB catalyze damage recognition and UvrC the damage excision process. The expression of *uvrA* and *uvrB* is induced upon induction of DNA damage as part of the SOS regulon in *E. coli*. The amount of UvrC is not dependent on the SOS response; it is present in less than ten molecules per cell. The amount of UvrA is induced tenfold in the presence of damage compared to 20–25 molecules in the uninduced state and that of UvrB increases from 250 to 1000 molecules (Van Houten *et al*, 2005). In spite of the low basal level of the proteins, it has been shown that on exposure to moderate

UV doses (also on exposure to sunlight), most if not all of the damage is removed by existing NER proteins. For instance, upon exposure to 10 J/m^2 UV-C 80% of the UV-induced lesions are removed by constitutive NER proteins; only the remaining 20% is excised by *de novo* synthesized proteins (Lin *et al*, 1997). Most of the NER studies have been dedicated to understanding the functioning of UvrA, UvrB and UvrC proteins in the damage recognition process and the interactions between the proteins. Less attention has been drawn to steps that follow damage excision.

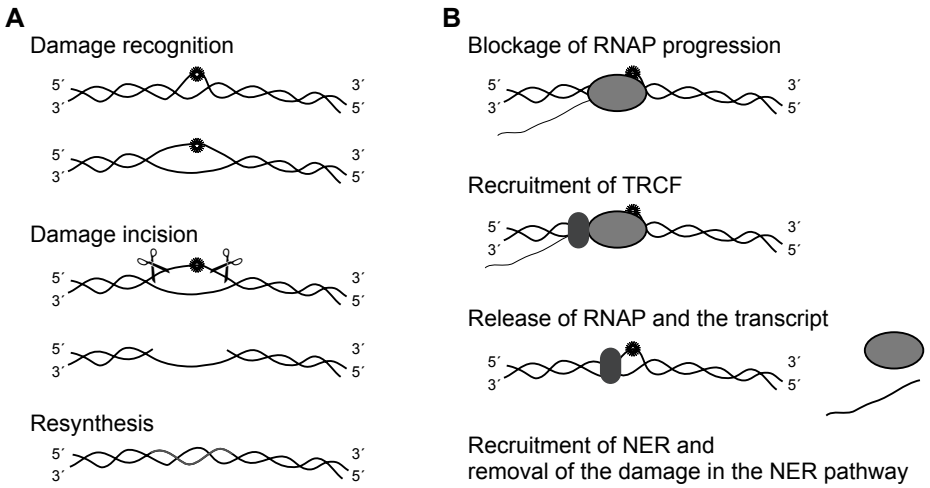


Figure 5. Schematic representation of nucleotide excision repair (NER). **A.** Main steps in the NER reaction. During DNA damage recognition DNA adjacent to the lesion is unwound. Damage is excised from the DNA in a short DNA fragment. The damaged oligonucleotide is removed and the resulting gap is resynthesized. **B.** Recognition of the damage in TC-NER. Progression of RNAP is blocked by DNA damage. Transcription-repair coupling factor (TRCF, Mfd) is recruited at the site of the blocked RNAP. TRCF releases the blocked RNAP, unfinished transcript and recruits NER at the damaged site. The damage is further processed according to the mechanism shown in panel A.

3.2.1. Damage recognition in nucleotide excision repair

Although NER has been studied *in vitro* over 20 years, it is still not clear how the damage is recognized in this pathway. The pathway is highly damage-specific, but can also occasionally incise undamaged DNA (Branum *et al*, 2001). As NER has been shown to repair structurally diverse damage, the general idea is that the damage is recognized by the structural changes that the adduct induces in the DNA. The distortion induced in the DNA backbone depends not only on the damage, but also on the thermostability of the DNA surrounding the lesion (Geacintov *et al*, 2002). In this way the sequence context influences

the conformation of the DNA containing the adduct and the efficiency of its recognition by NER. The NER damage recognition complex is a heterotetramer or heterotrimer consisting of the UvrA dimer and one or two molecules of UvrB. There is debate about whether UvrB is a monomer or a dimer in the complex. AFM, FRET and protein crosslinking studies suggest the presence of two UvrB molecules (Hildebrand & Grossman, 1999; Verhoeven *et al*, 2002b; Malta *et al*, 2007), while crystal structures and gel filtration suggest the presence of an UvrB monomer in the complex (Orren & Sancar, 1989; Machius *et al*, 1999; Theis *et al*, 1999; Truglio *et al*, 2006b). It cannot be ruled out that the composition of damage recognition complex varies among bacterial species. Recognition of the damage has been proposed to be a two-step process: first, damage is recognized by UvrA and thereafter verified by UvrB in the same protein complex. Successful recognition results in stable UvrB:DNA complex formation.

Damage localization by UvrA

Even though the $UvrA_2B_{(2)}$ complex has higher damage discrimination properties compared to UvrA alone, UvrA binds damaged DNA also in the absence of other NER proteins. Therefore most of the studies on NER damage recognition properties are carried out solely with UvrA (Truglio *et al*, 2006a). At physiological concentration UvrA forms dimers *in vitro* and the dimer is also the functionally relevant form of the protein present in the damage recognition complex together with $UvrB_{(2)}$. $UvrA_2$ binds preferentially to damaged DNA, with a specificity ratio of 1000:1 when compared to undamaged DNA (Van Houten *et al*, 1987). It has been suggested that $UvrA_2$ has two different stages of DNA binding: initial loose binding and stable binding achieved upon ATP hydrolysis (Strike & Rupp, 1985; Wagner *et al*, 2009). The loose binding possibly represents the damage localization process. It has been proposed that damage localization in the NER process occurs by a limited processive DNA scanning mechanism (Gruskin & Lloyd, 1988). Upon binding to damaged as well as undamaged DNA $UvrA_2$ probably bends and unwinds the DNA (Oh & Grossman, 1986). This could serve as a method to identify the 'abnormalities' in the DNA backbone. Recently it was confirmed that $UvrA_2$ indeed has two binding modes. The UvrA dimer in 'search' of the damage can probe simultaneously two DNA molecules, with one monomer bound to one DNA molecule and the other monomer to the other (Wagner *et al*, 2009). Upon damage recognition, both of the UvrA monomers in complex make contact with the lesion and form a stable $UvrA_2$:DNA complex. In order to form a stable complex with the damage, $UvrA_2$ has to undergo a conformational change that is possibly driven by ATP hydrolysis. $UvrA_2$ bound to the non-hydrolysable ATP analogue ATPγS is incapable of forming stable contacts with the damage due to the conformation of the protein that does not allow both of the UvrA monomers in the complex to make simultaneous contact with the damage (Wagner *et al*,

2009). Due to the inability to hydrolyze ATP the protein thus loses its damage discrimination properties (Seeberg & Steinum, 1982; Van Houten *et al*, 1988; Wagner *et al*, 2009). The same unspecificity in DNA binding is observed in the case of UvrA mutants defective in ATPase function (Thiagalingam & Grossman, 1991). If no damage is detected, protein dissociates without any cofactor usage. After initial damage detection by UvrA, damage verification by UvrB has to take place before a stable UvrB:DNA preincision complex can form.

The crystal structure of UvrA from *Bacillus stearothermophilus* reveals that UvrA forms dimers in asymmetric manner (Pakotiprapha *et al*, 2008). Although the protein was crystallized in the absence of DNA, it was possible to predict the DNA binding interface. On the surface of the dimer a positively charged concave was identified. Mutations in conserved amino acids in this area resulted in diminished DNA binding, confirming that the predictions were correct, and that the positively charged concave is indeed responsible for DNA binding (Croteau *et al*, 2008; Pakotiprapha *et al*, 2008). According to the structure, the DNA binding surface in the UvrA dimer could fit approximately 30 bp of DNA (Pakotiprapha *et al*, 2008). The same length was found protected in both footprint and AFM studies of UvrA bound to damaged DNA (Van Houten *et al*, 1987; Wagner *et al*, 2009).

Damage verification and UvrB preincision complex formation

After the damage is localized by UvrA and prior to its removal, the presence of the damage has to be verified by UvrB. UvrB in the $UvrA_2B_{(2)}$ complex scans the DNA by inserting its β-hairpin domain between the two strands of the DNA and moves along it trying to flip nucleotides out of the DNA helix (Theis *et al*, 1999; Skorvaga *et al*, 2004). The insertion of β-hairpin between the strands becomes possible due to previous DNA binding and local unwinding by UvrA. This so called limited DNA helicase or DNA destabilizing activity is dependent on UvrB ATPase activity that becomes exposed and activated in the presence of UvrA and the damaged DNA (Truglio *et al*, 2006a). During the damage verification process DNA is wrapped around the UvrB. ATP hydrolysis is needed for stable complex formation on damaged DNA as well as for release of the DNA on which no damage was detected (Verhoeven *et al*, 2001). As the base damage causes impaired base stacking, nucleotides adjacent to the damage are easily flipped out from the DNA, while others among undamaged nucleotides will stay in place due to their stacking. Since UvrB scans DNA in 3′-5′ direction, upon initial damage recognition by UvrB the base adjacent to the damage at the 3′ side is flipped out into an extrahelical conformation (Malta *et al*, 2006). When UvrB detects the damage, it hydrolyzes the bound ATP giving rise to its ADP-bound form (Moolenaar *et al*, 2000a). In that conformation UvrB does not yet form a stable complex with the DNA and the protein may dissociate. To form a stable UvrB:DNA complex, the ADP now associated with UvrB needs to be exchanged with ATP. A conformational

change concomitant with UvrB binding ATP then induces flipping out of the nucleotide in the undamaged strand opposite the base adjacent to the damage at the 3´ side (Malta *et al*, 2006). Flipping the nucleotide out in the undamaged strand is essential for later efficient damage excision by UvrC. The position of the damaged nucleotide itself does not change upon UvrB binding (Malta *et al*, 2008).

The DNA is given over from UvrA to UvrB only after damage verification (DellaVecchia *et al*, 2004). While probing DNA for damage using its DNA strand destabilizing activity, UvrB does not make direct contact with the DNA. Handover of DNA from UvrA to UvrB for stable UvrB:DNA complex formation is dependent upon ATP hydrolysis by UvrA (Truglio *et al*, 2006a). This process has been proposed to induce a conformational change in UvrB so that the protein becomes properly positioned on DNA with one of the DNA strands clamped between the β-hairpin and the domain 1b of the protein (Orren *et al*, 1992; Theis *et al*, 1999). Whether the DNA strand clamped is the damaged or undamaged strand is currently under debate. ATP hydrolysis by UvrA together with the stable UvrB:DNA complex formation induces $UvrA_2$ to dissociate.

As UvrB has only 3´-5´ polarity in scanning the DNA it can search for damage on only one DNA strand at the time. However, the presence of two UvrB molecules in the damage recognition complex might allow scanning of both DNA strands simultaneously (Moolenaar *et al*, 2000b; Verhoeven *et al*, 2002b). If no damage is recognized by one UvrB monomer, the protein might dissociate from DNA giving the possibility for the other monomer in the complex to scan the other DNA strand for damage. According to this scenario, as soon as the damage is located by one of the UvrB monomers, it will bind stably to the DNA. The other molecule remains loosely attached until UvrC joins the complex (Verhoeven *et al*, 2002b).

The sequential recognition process by UvrA and UvrB is thought to give the high damage specificity for the repair pathway. Not all DNA abnormalities identified as damage by UvrA are confirmed by UvrB and UvrB is not capable of binding to dsDNA by itself, but needs ATP-dependent loading by UvrA as described earlier. Not all lesions that result in stable UvrB:DNA complex formation are effectively excised by UvrC. This observation suggests that in addition to the two-step damage recognition process there are additional factors that determine the removal of a certain damage. Probably one of the extra criteria is how the lesion is exposed to UvrC in the UvrB:DNA preincision complex in different sequence contexts. It has been shown that DNA in the UvrB:DNA complex is heavily bent and that this bending is necessary for incisions to occur (Lin *et al*, 1992; Shi *et al*, 1992). This notion is further supported by the observation that the same damage in a different sequence context is excised from DNA with a different efficiency (Verhoeven *et al*, 2002a).

3.2.2. Damage excision and postincision steps

In order for NER reactions to proceed, the NER-specific endonuclease UvrC has to be recruited to the complex. UvrC and UvrA bind to the same domain of UvrB, domain 4. The usage of the same interaction domain ensures that UvrC does not bind UvrB and catalyze damage excision before UvrA has dissociated from the complex, i.e. before the damage recognition and verification steps are completed (Hsu *et al*, 1995). Damage is excised as a 12–13 nt DNA fragment, in a two step process mediated by two distinct endonuclease domains present in the N- and C-terminal domains of the protein. The UvrC N-terminal half is homologous to GIY-YIG superfamily endonucleases and the C-terminal half contains an endonuclease with no sequence homology to any other protein and DNA binding helix-hairpin-helix (HhH) domain. All of these structures have recently been solved separately (Singh *et al*, 2002; Truglio *et al*, 2005; Karakas *et al*, 2007). Structural comparisons revealed that C-terminal endonuclease domain of UvrC shares nevertheless structural homology with RNA endonuclease RNaseH. The HhH domain facilitates UvrC binding to DNA and is necessary to stabilize the UvrBC complex on DNA (Moolenaar *et al*, 1998). Depending on the lesion and the sequence context, occurrence of both of the incisions has been shown to need the presence of the DNA binding domain (Moolenaar *et al*, 1998; Verhoeven *et al*, 2002a). It has been proposed that the HhH domain is an additional factor responsible for UvrB:DNA preincision complex recognition by UvrC (Truglio *et al*, 2006a). The HhH domain binds preferentially to ssDNA-dsDNA junctions with bubble structures of at least six unpaired basepairs, which is the structure of DNA in the preincision complex (Singh *et al*, 2002). Incisions at either side of the damage catalyzed by the endonuclease domains are not carried out simultaneously. 5′ incision is catalyzed by the C-terminal endonuclease domain only after the 3′ incision by the N-terminal endonuclease domain or in the presence of pre-existing nick. It has been proposed that the UvrBC complex on DNA in fact has two different DNA binding modes depending on the incision taking place (Zou & Van Houten, 1999). For the first incision UvrBC complex binds a dsDNA region three bases 3′ of the damage site. The incision is catalyzed at the fourth or fifth phosphodiester bond 3′ to the damage. The resulting nick relieves the DNA stress and leads to further opening of the DNA strands. The HhH domain of UvrC is necessary to stabilize the protein complex on DNA after the first incision (Moolenaar *et al*, 1998). The 5′ incision is catalyzed immediately after 3′ incision by the endonuclease domain in the C-terminal half of UvrC. The second incision occurs independently of UvrB interaction, at the eight phosphodiester bond 5′ to the damage (Lin *et al*, 1992; Lin & Sancar, 1992).

The oligomeric state of UvrC is not known. In solution *E. coli* and *Bacillus caldotenax* UvrC exists as monomers and tetramers (Nazimiec *et al*, 2001; Tang *et al*, 2001). It was shown that the UvrC monomer makes both of the incisions after the damage is recognized by UvrA and UvrB. The tetrameric form has been suggested to exhibit catalytic activity as well, but it mediates 5′ incision

only. The UvrC tetramer can also bind DNA damage independently of $UvrA_2B_{(2)}$, suggesting that the protein may have a NER-independent capability to promote damage removal (Nazimiec *et al*, 2001).

E. coli and some other species encode a protein, Cho, that is homologous to the N-terminal half of UvrC. Cho can perform only 3′ incision; the phosphodiester bond cleavage is catalyzed four nucleotides further away compared to UvrC (Moolenaar *et al*, 2002). Similarly to UvrC, the protein interacts with UvrB, but the nature of the interaction is not known. 3′ incision by Cho is not dependent of domain 4 of UvrB, indicating a different mode of binding and incision. The incision efficiencies of the UvrC N-terminal GIY-YIG domain and Cho vary. It has been proposed that Cho is important in incising the DNA 3′ of the large adducts that cannot be incised by UvrC due to steric hindrance. After 3′ incision catalyzed by Cho, the 5′ cut still needs to be carried out by UvrC. However, Cho proteins from some species encode an additional domain that has homology to proofreading subunit of DNA polymerase III. Therefore it has been proposed that these proteins can remove the damaged nucleotide from DNA without UvrC, but with its 3′-5′ exonuclease activity (Goosen & Moolenaar, 2008).

After damage excision, a DNA fragment containing the damage is released by UvrD helicase and the resulting gap is filled by DNA pol I. UvrD and DNA pol I have been shown to release UvrB and UvrC from the DNA (Caron *et al*, 1985; Husain *et al*, 1985). UvrD has been proposed to release the damaged oligomer and UvrC from the complex, making repair synthesis possible and DNA polymerase I displaces UvrB bound to the gapped DNA (Orren *et al*, 1992). Exact mechanisms of these reactions are unclear. The NER pathway is completed by joining the newly synthesized DNA to the parent DNA by DNA ligase I.

3.2.3. Transcription-coupled NER

UV-induced lesions are repaired approximately tenfold more rapidly in actively transcribed genes than in non-transcribed ones with repair occurring preferentially on the transcribed strand (Bohr *et al*, 1985; Mellon & Hanawalt, 1989; Terleth *et al*, 1989). This phenomenon of strand-specific DNA repair is dependent on transcription-repair coupling factor Mfd (also referred as TRCF). *E. coli* Mfd is a 130-kDa monomeric protein comprised of eight domains, three of which (1a, 2 and 1b) show a high degree of structural homology with the UvrB protein (Assenmacher *et al*, 2006; Deaconescu *et al*, 2006). These are the domains responsible for UvrA recruitment to the site of stalled RNA polymerase. Mfd recognizes RNA polymerase which elongation is blocked due to the presence of DNA damage (Selby & Sancar, 1993). It dissociates stalled transcription complexes by pushing RNAP forward along the DNA in an ATP-dependent fashion (Park *et al*, 2002). RNAP displacement requires the

interaction of Mfd with the β subunit of stalled RNAP and DNA immediately upstream of the RNAP (Park *et al*, 2002; Smith *et al*, 2007). Pushing RNAP forward by Mfd with its DNA translocation activity is thought to destabilize the transcription complex by unwinding the DNA–RNA hybrid and rewinding the single-stranded transcription bubble (Park & Roberts, 2006). Mfd still bound to the DNA recruits NER by binding to UvrA (Selby & Sancar, 1993; Assenmacher *et al*, 2006). Mfd-UvrA interaction is shown to be responsible for the enhanced rate of repair that is observed during transcription-coupled repair (Selby & Sancar, 1995). After the recruitment of UvrA to the damaged site by Mfd, the remainder of NER reactions will take place as described previously.

3.3. Pyrimidine dimer-DNA glycosylase

There are several UV-induced damage repair pathways that are unique to a subset of bacterial species. These so called alternative pathways exist mostly in bacteria that lack previously characterized enzymatic photoreactivation activity and therefore provide functional complementation. Repair by pyrimidine dimer-DNA glycosylases (PD-DNA glycosylases) is one of these less common mechanisms to remove UV-induced damage. This mechanism has so far been found in *Micrococcus luteus* and in several *Bacillus*, *Neisseria* and *Haemophilus* species (Nyaga & Lloyd, 2000; Vasquez *et al*, 2000; Lloyd, 2005). PD-DNA glycosylases are enzymes that remove UV-induced pyrimidine dimers from DNA, resulting in a single nucleotide or longer gap in the DNA that needs to be filled in by DNA polymerase. Based on the mechanism by which lesions are removed, PD-DNA glycosylases are classified as base excision repair (BER) pathway enzymes.

BER consists of several DNA glycosylases and glycosylase/AP lyases that recognize only particular types of DNA damage. Most glycosylases recognize specific types of endogenous DNA damage i.e. oxidized and alkylated nucleobases. Regardless of the damage recognized by a specific glycosylase, the mechanism of its removal from the DNA is the same. First, the damaged nucleobase is excised by hydrolysis of N-glycosyl bond between deoxyribose and nitrogen base, leaving behind an apurinic/apyrimidinic site (abasic site/AP site). Subsequently a nick is introduced to DNA in the immediate vicinity of the AP site either by a separate enzyme, AP endonuclease or by AP lyase activity of the glycosylase/AP lyase. For the removal of an AP site and concurrent generation of a single nucleotide gap, further action of exonucleases and DNA-deoxyribophosphodiesterase (dRpase) or 3′ phosphodiesterase is needed, depending on whether the nick next to the AP site was induced by AP endonuclease or glycosylase/AP lyase, respectively. The BER reaction is completed by filling in the missing nucleotide by DNA pol I and covalent joining by ligase. Alternatively, exonucleases may extend the DNA gap by removing additional nucleotides from the DNA, or DNA pol I may synthesise more than one nucleotide creating a 5′ flap by its strand displacement activity. In these

cases, removal and resynthesis of several nucleotides occurs. This process is referred to as 'long patch BER', in contrast to 'short patch BER' during which a single nucleotide is removed and resynthesized during the repair reaction (Friedberg *et al*, 2006).

The best characterized PD-DNA glycosylase in bacteria is *E. coli* phage T4 pdg. In describing the mechanism of action of PD-DNA glycosylases I will mainly rely on data available for this protein. Unfortunately the *in vivo* data on these enzymes is scarce and therefore it is hard to estimate the importance of PD-DNA glycosylases in UV-induced damage repair. PD-DNA glycosylases recognize only CPDs; glycosylases that recognize 6-4PPs have not been found so far (Lloyd, 2005). Damage recognition by PD-DNA glycosylases involves scanning of the DNA. The proteins bind to undamaged DNA by random collision. Once bound to DNA, the enzymes move along the molecule introducing nicks at the sites of CPDs (Lloyd *et al*, 1980; Ganesan *et al*, 1986; Gruskin & Lloyd, 1986). PD-DNA glycosylases are glycosylase/AP lyases and catalyze removal of a damaged base and cleavage of the phosphodiester backbone next to the resulting AP site. The AP lyase activity allows PD-DNA glycosylases also to recognize and cleave the DNA containing abasic sites (Nyaga & Lloyd, 2000). From CPD adducts consisting of two pyrimidines, only the N-glycosyl bond of the pyrimidine at the 5′ side is hydrolyzed (Figure 6). Since the bond cleavage does not affect the cyclobutane ring connecting the nitrogen bases of the CPD, the reaction does not lead to the release of the free nucleobase as common for the BER reaction. Instead, pyrimidine remains connected to the adjacent base via the cyclobutane ring. To initiate the removal of baseless deoxyribose PD-DNA glycosylase induces cleavage of the phosphodiester backbone 3′ of the AP site with its AP lyase activity (Lloyd, 2005). The subsequent steps in CPD removal from the DNA are not so well established. It has been reported that the monomerization of CPDs can occur in photoreactivation reactions. In that case the 3′ pyrimidine within the CPD is not removed from the DNA, but instead the AP site is removed by 3′ phosphodiesterase and the resulting single nucleotide gap is filled by DNA pol I. Most likely the other nucleotide forming CPD is removed from DNA by 5′-3′ exonuclease activity, resulting in extended DNA gap (Friedberg *et al*, 2006).

Solving the crystal structure of T4 pdg has shed more light on the mechanisms of PD-DNA glycosylase action. Catalytic sites for both reactions reside in the N-terminus of the protein (Doi *et al*, 1992; Hori *et al*, 1992). During both N-glycosyl bond cleavage and lyase reactions a covalent bond is formed between C1 of the 5′ deoxyribose in CPD and alpha-amino group of the N-terminal threonine residue. The nucleotide opposite the damage or AP site is flipped out to provide space for the reactions (Vassylyev *et al*, 1995; Golan *et al*, 2006).

3.4. UV-damage endonuclease

UV-damage endonuclease (UVDE) constitutes an alternative UV-induced damage repair pathway found in bacteria only recently (Takao *et al*, 1996; Earl *et al*, 2002). Homologues of this nuclease, well characterized in fungi, have so far been identified in 30 bacterial genomes, mostly in *Bacillus*, *Clostridium* and *Deinococcus spp*. (Goosen & Moolenaar, 2008). In *Deinococcus radiodurans* UVDE fully complements NER in removal of UV-induced DNA damage, recognizing equally well CPDs and 6-4PPs (Tanaka *et al*, 2005). This finding suggests that UVDE constitutes an extremely efficient repair pathway. Similarily to NER, UVDE can remove a vast variety of different lesions from DNA. UVDE removes a lesion by introducing a nick immediately 5′ of the CPD or 6-4PP, creating 3′ OH and 5′ phosphate termini (Figure 6). It is hypothesized that following the cleavage strand displacement synthesis occurs from the nicked site (Yonemasu *et al*, 1997). This results in the formation of a 5′ flap structure, which is removed by the flap endonuclease activity of DNA pol I carrying out the repair synthesis or by a separate flap endonuclease.

Recently, the crystal structure of UVDE of *Thermus thermophilus* was solved (Paspaleva *et al*, 2007). UVDE is a monomeric globular protein, containing a deep groove with catalytic site located in the bottom of the groove. Although UVDE was crystallized without the DNA, its structural homology with Endo IV allowes to predict that damaged nucleotides are flipped out to this cavity upon the DNA binding. Mutating conserved amino acids in this region made the protein incapable of incising the damaged DNA, indicating that residues in the cavity are important in damage recognition. Conformational similarities with Endo IV suggest that in addition to the damaged nucleotides, the undamaged nucleotides on opposing strand will also be flipped out during the phosphodiester bond hydrolysis (Paspaleva *et al*, 2007).

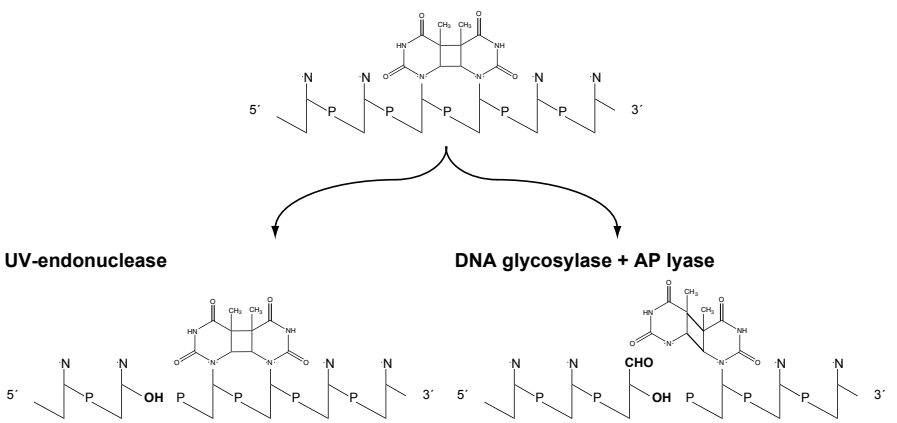


Figure 6. Removal of CPD by pyrimidine dimer-DNA glycosylase and UV-damage endonuclease.

4. Tolerance to UV-induced DNA damage

UV-induced DNA damage can be repaired by various repair systems as described in the previous section. However, cells have also evolved a variety of other strategies for coping with damaged DNA. Differently from DNA repair, these mechanisms do not result in removal of the damage and are therefore referred to as damage tolerance mechanisms. These mechanisms possibly become important in conditions of excessive DNA damage. Damage tolerance mechanisms are based on normal cellular processes: homologous recombination (single-strand DNA gap filling) and DNA synthesis (translesion synthesis, TLS).

4.1. Single-strand DNA gap-filling

DNA synthesis may be discontinuous due to the presence of the DNA damage, leaving gaps in the nascent strands (Heller & Marians, 2006). It has been shown that such ssDNA gaps are generally repaired by recombination in the RecF pathway (Tseng *et al*, 1994). The RecF pathway involves the action of RecA, RecF, RecO, RecR and SSB proteins as well as the proteins participating directly in the homologous recombination pathway (RecG, RecJ, RecN, RecQ, RuvA, RuvB, RuvC) (Kowalczykowski *et al*, 1994).

ssDNA regions (gaps) occurring in cells are coated with single-strand binding proteins (SSB) to protect them from being digested by nucleases. In order to permit any recombinational events to occur, SSB bound to ssDNA has to be substituted with RecA. RecA is not capable of outcompeting SSB by itself, but needs to be loaded onto the DNA. On a gapped DNA substrate, RecF, RecO and RecR proteins are important in this process (Morimatsu & Kowalczykowski, 2003). RecA can also be loaded onto the DNA *via* the RecBCD pathway, but as this pathway is not relevant in terms of ssDNA gap repair, it will not be discussed here.

Loading and stabilizing the RecA nucleoprotein filament by the RecFOR proteins has been observed only when the substrate is gapped DNA. The stochiometry of the proteins involved and the exact sequential order of the events are poorly understood. RecF has been shown to bind specifically to gapped DNA at the 5′ end of the gap at the double-strand DNA (dsDNA) - ssDNA junction (Hegde *et al*, 1996; Morimatsu & Kowalczykowski, 2003). It has been suggested that as initial step RecF or RecFR complex binds to the gap. Subsequently, RecOR or RecO joins the preexisting complex and possibly causes changes in the conformation of the ssDNA-SSB protein complex and/or displaces some SSB molecules so that RecA can bind. The RecA protein filament then cooperatively extends over the entire gapped ssDNA region in 5′ to 3′ direction (Morimatsu & Kowalczykowski, 2003). This process as well as preventing the dissociation of RecA from ssDNA ends is facilitated by RecOR (Inoue *et al*, 2008). A RecA nucleoprotein filament formed on gapped DNA is able to interact with dsDNA and to search for a homologous DNA region. If

homology is found, the RecA-coated ssDNA strand and the dsDNA strand become noncovalently joined by hydrogen bonding between the nucleobases. For recombination events to proceed, a nick has to be introduced into one of the DNA molecules: either the ssDNA or the homologous dsDNA. It has been shown that the nick is introduced into the dsDNA specifically, opposite the ssDNA gap; the enzyme responsible for that activity is not known (Friedberg *et al*, 2006). Subsequently, DNA strand exchange takes place and an intermediate in the recombination reaction, a so called Holliday junction is formed. In this structure branch migration catalyzed by RecA, RuvAB and RecG proteins takes place. As a result the ssDNA gap becomes aligned with the complementary undamaged DNA strand and the gap is filled by replication. Thereafter the Holliday junction is resolved by cleavage mediated by RuvC or reverse branch migration catalyzed by RecG and RuvAB. This recombinational gap repair thus allows filling the ssDNA gaps that have emerged due to DNA damage, using intact DNA as a template. Nevertheless, in this process DNA damage *per se* is not removed from DNA. For that purpose DNA repair by any of the previously described mechanisms has to take place.

At the moment the discontinuous replication model is generally accepted and therewith the formation of ssDNA gaps due to encountered DNA damage. However, not all research groups have reached the same conclusion. There are still doubts whether ssDNA gaps are at all formed during replication and if they form, whether they are repaired by recombinational events. For instance, genetic studies by the group of Robert G. Lloyd (Mahdi *et al*, 2006) suggest that if ssDNA gaps are formed at all, the recombination is not an essential mechanism in their removal. Instead filling in the gaps may be carried out by the specialized DNA polymerases DNA pol II, DNA pol IV or DNA pol V, although neither of these DNA polymerases is specifically needed for gap closure. It can also not be excluded that gaps are formed but filled in by DNA pol I in the same way as the polymerase helps to complete lagging strand synthesis (Mahdi *et al*, 2006).

4.2. Translesion synthesis

Translesion DNA synthesis (TLS) is a process in which DNA polymerase carries out replication across a DNA lesion. DNA polymerase inserts either a ‘correct’ or a ‘random’ nucleotide opposite the lesion, skips the lesion or adds more nucleotides opposite the lesion than necessary. The process can therefore be either accurate or result in base substitutions or frameshift mutations in replicated DNA. Not all DNA polymerases are capable of TLS. Mostly TLS is carried out by specialized DNA polymerases that are induced in response to DNA damage (SOS response). Many of them belong to the ‘Y-family’ of DNA polymerases (Ohmori *et al*, 2001). For instance *E. coli* possesses three specialized DNA polymerases: DNA pol II, DNA pol IV and DNA pol V. The last two mentioned are members of the Y-family DNA polymerases. The

properties that differentiate the Y family DNA polymerases from all other polymerases and that make them suitable for TLS will be discussed later. Not all TLS DNA polymerases synthesize across the same types of lesions. There are differences in 'substrate' specificity and also in the manner in which different lesions are dealt with (Wagner *et al*, 2002). For instance, DNA pol II or DNA pol V is needed to synthesize across the synthetic compound *N*-2-acetylaminofluorene (AAF) when present in DNA. In the same sequence context synthesis by DNA pol II results in a -2 deletion, but is error-free in the case of DNA pol V (Napolitano *et al*, 2000). Replication across UV-induced DNA damage has been shown to be dependent on DNA pol V in *E. coli*. The polymerase is discussed in detail in the next section. As DNA replication by specialized DNA polymerases can in general be error-prone on damaged as well as on undamaged DNA, their involvement in DNA replication has to be controlled. Access of specialized DNA polymerases to DNA replication will be discussed next.

As mentioned before, replicative DNA polymerases stall upon encountering a DNA damage as they are not capable of TLS. For TLS to occur (1) replicative DNA polymerase (DNA pol III) blocked on a lesion has to dissociate or free the replication fork and (2) a specialized DNA polymerase needs to be recruited. Specialized DNA polymerases interact with the β sliding clamp: the processivity factor of the replication machinery as mentioned earlier (Tang *et al*, 1999; Dalrymple *et al*, 2001; Indiani *et al*, 2005). It has been speculated that one β-clamp could physically simultaneously accommodate two different DNA polymerases, a replicative one and a specialized polymerase (Pagès & Fuchs, 2002). A model has been proposed in which specialized DNA polymerases are connected to the replication machinery *via* the β-clamp and used as 'tools on a tool belt'. In this way specialized DNA polymerases could transiently take over the DNA synthesis when the replicative DNA polymerase stalls to bypass the damage without affecting much the overall speed of the replication process. The fact that DNA pol III and pol IV are able to interact simultaneously with the same β-clamp supports this idea (Indiani *et al*, 2005). It has been shown that switching between the replicative polymerase and DNA pol IV occurs rapidly, but DNA pol IV does not get involved in the replication unless DNA pol III stalls. After the damage is overcome, DNA synthesis is rapidly switched from pol IV back to pol III (Indiani *et al*, 2005). In addition to interaction *via* the β-clamp, DNA polymerases III and IV are proposed to have direct interaction as well. This interaction further modulates the exchange between DNA pol III and pol IV at the replication fork (Furukohri *et al*, 2008). Recent findings suggest that specialized DNA polymerases DNA pol II and DNA pol IV are not dependent in their DNA synthesis on stalling of replicative DNA polymerase but they efficiently switch with DNA pol III also in moving replisome. This switching is dependent on the intermediate where two DNA polymerases are simultaneously attached to the same β-clamp. Replisomes containing DNA pol II or DNA pol IV are stable and capable of synthesizing thousands of base-pairs of DNA without dissociation (Indiani *et al*, 2009). These dynamic interactions with replicative DNA

polymerase have so far not been described for DNA pol V. Whether similar relations exist is not known, but as DNA pol V interacts with the β-clamp in the same manner as other specialized DNA polymerases, it is likely.

DNA replication by specialized DNA polymerases under conditions of DNA damage has been getting attention as having yet another role in addition to carrying out TLS across lesions. DNA pol II and pol IV replisomes have a three- to tenfold reduced replication speed compared to DNA pol III. Therefore it is hypothesized that replication by DNA pol II or DNA pol IV could serve as a molecular brake to allow time to repair the DNA before the replication fork reaches the lesion (Indiani *et al*, 2009; Langston *et al*, 2009). Although similar observations have also been made *in vivo* when over-expressing DNA polymerases IV or II (Uchida *et al*, 2008; Indiani *et al*, 2009), the relevance of this type of check-point is questionable as deletion of these polymerases has relatively minor effect on viability of bacteria compared to their effect on mutagenesis (Courcelle, 2009).

All specialized DNA polymerases can share the β-clamp with DNA pol III. The ability of the β-clamp to bind two specialized DNA polymerases simultaneously explains elegantly how specialized DNA polymerases could be recruited to TLS during the replication process. The particular additional DNA polymerase attached to the β-clamp at a given moment could be dependent on instant cellular conditions.

Characteristics of the Y family DNA polymerases

TLS across different types of lesions is often carried out by specialized DNA polymerases belonging to the Y family of DNA polymerases. These DNA polymerases, widely spread in bacteria as well as in eukaryotes and archea share specific characteristics that will be discussed in this section. *E. coli* possesses two Y family DNA polymerases: DNA pol IV and DNA pol V. DNA pol V carries out DNA synthesis across UV-induced DNA adducts and is responsible for elevated mutation rate upon exposure to UV. The *dinB*-encoded DNA pol IV does not contribute to UV-induced mutagenesis but elevates spontaneous mutation frequency when overexpressed in log-phase bacteria (Kim *et al*, 1997; Wagner & Nohmi, 2000) and is involved in mutagenesis in stationary-phase bacteria (McKenzie *et al*, 2001; Galhardo *et al*, 2009). Y family polymerases exhibit low fidelity and low processivity. The low fidelity is partly a consequence of the lack of 3´-5´ exonuclease proofreading activity, which is present in the replicative polymerases. Moreover it is ascribed to the active site that allows non-canonical base pair formation (Ling *et al*, 2001; Silvian *et al*, 2001; Zhou *et al*, 2001). Comparison of crystal structures of low and high-fidelity DNA polymerases (Dpo4 from *Sulfolobus solfataricus* (Y family) and DNA pol I from *Bacillus stearothermophilus* (A-family) sheds some light on this property (Figure 7) (Broyde *et al*, 2008). The first 250-350 N-terminal amino acid residues of all

known DNA and RNA polymerases constitute the catalytic core of the polymerases and form the so called 'thumb', 'palm' and 'finger' subdomains (Kulaeva *et al*, 1996). The palm domain is highly conserved, but the secondary structures of the finger and the thumb domains vary broadly across different polymerase families (Yang, 2005). These are the domains responsible for DNA and nucleotide substrate interaction. The thumb and the finger domains of the Y family polymerases are distinctly smaller compared to the same domains in all other classes of DNA polymerases. The smaller size of these domains in Y family DNA polymerases does not permit detection of the formed mismatch in their active site. Incorporation of a non complimentary nucleotide in the nascent DNA strand causes conformational changes in the primer-template duplex. In the case of polymerases from other families than Y, this change impedes the incorporation of the next nucleotide as the primer and the nucleotide cannot be aligned to the polymerase active site for phosphodiester bond formation. Y family DNA polymerases have a unique conserved 'little finger' domain that together with the thumb facilitates the movement of the primer template duplex in the active site (Ling *et al*, 2001). The more flexible the little finger and thumb are, the more distortion in the primer template confirmation is allowed for the next nucleotide to be incorporated. As the mobility of the little finger and the thumb alters the position of the DNA substrate relative to the catalytic core, it also influences the activity of the polymerase (Ling *et al*, 2003; Rechkoblit *et al*, 2006). Y family polymerases do not incorporate fully random nucleotides in nascent DNA, but are selective and exhibit a certain fidelity. Fidelity of the Y family polymerases depends on formation of hydrogen bonds between the DNA and the incorporated nucleotide. The size or the shape of the base pair formed is less important (Mizukami *et al*, 2006; Potapova *et al*, 2006). Errors in replication that still arise are thus mostly due to changed hydrogen bonding of damaged nucleotides.

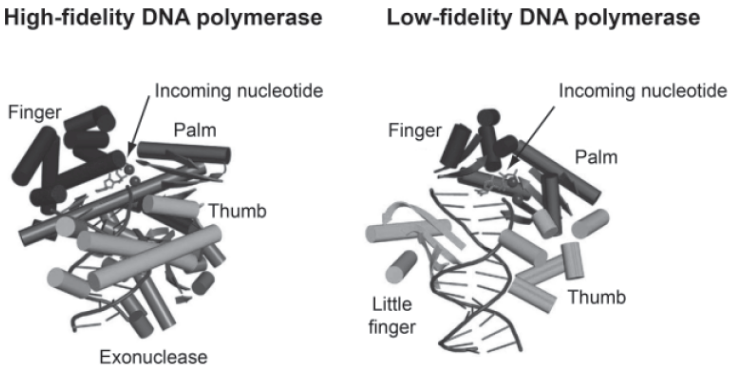


Figure 7. Schematic representation of active sites of high-fidelity versus low-fidelity DNA polymerases. As example of high-fidelity polymerases *B. stearothermophilus* DNA pol I (**A.**) and of low-fidelity polymerases Dpo4 from *S. solfataricus* (**B.**) are shown. The main subdomains forming the catalytic core of the polymerases: 'thumb', 'palm' and 'fingers' as well as the Y family DNA polymerase-specific 'little finger' subdomain are indicated. Figure adapted from Jiang *et al* (2009a)

4.2.1. *E. coli* DNA polymerase V

It was noticed already several tens of years ago that *E. coli* cells exposed to UV radiation have a significantly elevated mutation frequency (Bridges, 1966). This highly mutable phenotype was shown to be dependent on *recA* and *lexA*, as no increase in mutations was detected in their deficient or defective backgrounds (Schlacher & Goodman, 2007). *lexA* deficiency results in cell death unless the cell division inhibitor *sulA* is also absent. Therefore the mutants considered as *lexA*$^{-}$ were actually deficient in LexA autocatalytic cleavage activity and therefore did not have any SOS induction (See Figure 3 for SOS response scheme). These mutants are further referred to as *lexA*$^{ind-}$. Mutability was not the only phenotypic effect that was not observed in *recA*$^{-}$ or *lexA*$^{ind-}$ strains upon UV irradiation compared to the proficient strains: mutants were also deficient in λ prophage reactivation and SulA-dependent cell filamentation. These results suggested indirect participation of RecA and LexA in UV mutagenesis (Friedberg *et al*, 2006). At present, when knowing more about the nature of the SOS response, one would easily reach the conclusion, that the factors causing UV mutagenesis have to be controlled by this previously described network. After genetic screens to find the mutants lacking UV-induced mutagenesis but still capable of λ activation and filamentation upon UV exposure, the mutagenic phenotype was directly assigned to *umuD* and *umuC* products (Kato & Shinoura, 1977). Mutations in both of these genes resulted in the same non-mutable modestly sensitive phenotype in response to UV exposure (Elledge & Walker, 1983). Still, both *umu*$^{-}$ strains were much less sensitive to UV than *uvr*, *recA* or *lexA* strains (Bagg *et al*, 1981). Participation of the *umuD* and *umuC* products in UV-induced mutagenesis and UV resistance suggested that their role was connected to replication across the UV damage. It was believed for a long time that UmuD and UmuC assist the replicative DNA polymerase in UV damage bypass. A two-step model was proposed in which DNA pol III incorporates a nucleotide opposite the UV lesion, but cannot continue extending the DNA without the assistance of UmuD and UmuC (Bridges & Woodgate, 1985; Rajagopalan *et al*, 1992). Only ten years later, these proteins were purified and shown to be capable of carrying out DNA synthesis without the presence of DNA pol III in a DNA replication assay (Bruck *et al*, 1996; Reuven *et al*, 1999; Tang *et al*, 1999). Hence the product of the *umuD* and *umuC* genes was designated DNA pol V.

The *umuDC* genes encoding DNA pol V belong to the SOS regulon and are some of the last genes expressed after the induction of SOS signal. It has been shown that there are 400 molecules of UmuD and only 10-60 molecules of UmuC after SOS induction. Functional DNA pol V is UmuD′$_2$C, composed of three subunits: 46 kDa UmuC and two 12 kDa UmuD′ subunits. The active site of the polymerase locates in UmuC, but in the absence of UmuD′ subunits it is unable to carry out DNA synthesis. UmuD′ is necessary for providing proper contact with the β-clamp, the processivity factor of replication (Duzen *et al*, 2004). To form a heterotrimer with polymerase activity UmuD needs to undergo

a self-cleavage reaction mediated by the RecA nucleoprotein filament. UmuC associated with intact UmuD does not have a polymerase activity, but instead acts as a DNA damage checkpoint by directly blocking DNA replication. This feature is possibly needed for delaying mutagenic DNA pol V-mediated TLS for error-free repair. UmuD cleavage has been proposed to serve as a molecular switch that allows TLS of unrepaired or irreparable lesions. UmuD has also been shown to be a negative regulator for DNA pol V. It can out-compete UmuD′ from UmuD′$_2$C complex thus leading to UmuD′D heterodimer formation, which precipitates and inactivates the polymerase (Sutton *et al*, 2000). The mechanism is used for shutting off the DNA pol V activity if the damage has been repaired and RecA* to convert UmuD to UmuD′ is not available anymore.

DNA pol V carries out DNA synthesis across various UV-induced DNA adducts. It has been shown to be relatively accurate when replicating over T-T CPDs, as it preferentially incorporates dA opposite the lesion (Lawrence *et al*, 1990; Smith *et al*, 1996; Tang *et al*, 2000). The synthesis across T-T 6-4PP on the contrary is mutagenic and dG is frequently incorporated opposite the 3′T (LeClerc *et al*, 1991; Smith *et al*, 1996; Tang *et al*, 2000). Most common mutations arising from DNA pol V mediated bypass of UV-induced DNA adducts are T→A transversions and T→C transitions, with transversions being fivefold more frequent than transitions. While replicating undamaged DNA, DNA pol V makes base substitution errors with a frequency of 10^{-2}–10^{-4}, compared to 10^{-6}-10^{-7} of DNA pol III (Tang *et al*, 2000; Yang & Woodgate, 2007).

Catalytic activity of DNA polymerase V

Y-family DNA polymerases are intrinsically error-prone, and therefore it is essential for a cell to strictly regulate their expression and activity. In this way the risk of any unwanted mutagenesis is minimized and it is ensured that the polymerase is used for replication only at a specific time and location. DNA pol V is regulated at both transcriptional and posttranslational level. In addition, its activity is regulated by modulating its access to the replication fork, but that is not all. The catalytic activity of DNA pol V is also controlled. All these different levels of control are needed to ensure that the polymerase carries out DNA synthesis only if there is a real need for that.

DNA pol V needs accessory proteins to carry out DNA synthesis. It has been shown that *in vivo* DNA synthesis by DNA pol V is dependent on RecA and the β-clamp and possibly SSB is also needed (Becherel *et al*, 2002). Neither the β-clamp nor SSB are essential for TLS *in vitro* (Pham *et al*, 2002; Fujii *et al*, 2004), but their presence enhances the replication efficiency under specific assay constraints (Tang *et al*, 1999; Pham *et al*, 2001; Maor-Shoshani & Livneh, 2002). DNA pol V is unable to synthesize DNA in the absence of RecA in *in vivo* and *in vitro* conditions (Pham *et al*, 2001; Pham *et al*, 2002; Fujii *et*

al, 2004). Although the need for RecA by DNA pol V has never been under question, it has been a long time mystery in what form RecA is needed. Earlier models suggested that RecA* formed directly on the damaged DNA strand was needed for DNA pol V activity. It was initially suggested that RecA assisted the polymerase in its positioning on the lesion (Echols & Goodman, 1990). The finding that interaction with the 3′ proximal end of the filament is crucial for DNA pol V activity only strengthened this model. The proposed direct need for RecA* *in cis* was not consistent with the results of further experiments, demonstrating that the RecA filament in front of the replication fork was an obstacle blocking the movement of DNA polymerase V. Replication was resumed when SSB was added to the reaction to destabilize the RecA filament (Pham *et al*, 2001). This observation led to a new model, according to which RecA was actively removed in front of the moving DNA polymerase (the so called 'Cowcatcher' model (Pham *et al*, 2001)). However, there were still data not fitting the model: replication by DNA pol V was observed even when the template strands too short to support RecA filament assembly were used in the assay (Schlacher *et al*, 2005). This finding suggested that for activation of the polymerase, formation of RecA filament in *cis* was not essential; still a direct interaction of pol V with the 3′-proximal tip of RecA was required for the polymerase activity. Further studies confirmed that DNA pol V interacted with 3′ end of RecA filament formed *in trans*, not on the DNA template strand being copied (Schlacher *et al*, 2006). Recently it was found that interaction of DNA pol V with RecA* results in transfer of the 3′-proximal RecA-ATP subunit to the polymerase. Thereafter the polymerase is active to carry out DNA synthesis on any detected template. After one round of replication DNA pol V is inactivated and needs to encounter RecA* to exchange RecA-ATP (Jiang *et al*, 2009a).

The finding that DNA pol V is activated by RecA* *in trans* also gives more information about DNA pol V-mediated TLS. TLS has been considered as especially important if DNA lesions are closely spaced and on opposite DNA strands. The main reason for this is that closely located lesions are bad substrates for repair mechanisms such as NER and recombinational repair. Consistent with the RecA* *in trans* model, RecA* on one strand (ssDNA gap) could activate DNA pol V for TLS in the opposite gap. Thereafter the DNA lesion can be removed in the NER pathway and the remaining gap on the opposite strand can be removed in the recombination process (Schlacher & Goodman, 2007).

umuDC orthologs in other bacteria

Most bacteria do not have *umuDC* genes encoding DNA polymerase V in their chromosomes. Actually, besides *E. coli* chromosomally encoded DNA pol V has been found only in *Salmonella typhimurium* and *Shigella flexneri* (Runyen-Janecky *et al*, 1999). Nevertheless, non-chromosomal *umuDC* orthologs are

described in various bacteria, mostly encoded in self-transmissible broad host range plasmids (Permina *et al*, 2002). Based on amino acid sequence conservation UmuDC proteins encoded in mobile elements are grouped as UmuDC, MucAB, ImpAB, RumAB and RulAB. In addition to similarity at the amino acid level, most of the *umuDC* orthologs complement *E. coli umuDC*-deficient strains in UV survival and SOS-induced mutagenesis (Koch *et al*, 2000). All *umuDC* operons described to date are regulated by LexA and the encoded UmuD subunit undergoes RecA* mediated autocatalytic cleavage (Runyen-Janecky *et al*, 1999).

umuDC orthologs are widely distributed in naturally occurring isolates of *Pseudomonas* species, especially in those with phyllospheric habitat (Sundin *et al*, 1996; Sundin & Murillo, 1999; Sundin *et al*, 2004a; Zhang & Sundin, 2004a; Zhang & Sundin, 2004b; Cazorla *et al*, 2008). The strains harboring the RulAB-encoding plasmids have increased survival and mutability when exposed to UV-C or to simultaneous UV-A and UV-B radiation. Several different *umuDC* orthologs have been described in *Salmonella typhimurium*: SamAB on 60-MDa virulence plasmid, ImpAB on TP110 and MucAB on R46 plasmid (Nohmi *et al*, 1991). Plasmid encoded *umuDC* orthologs have been found also in *Proteus rettgeri*, *Serratia marcescens* and *Shigella flexneri* (Kulaeva *et al*, 1998; Runyen-Janecky *et al*, 1999).

There are only few data available on UmuC proteins in gram-positive bacteria. Differently from gram-negative bacteria described above, no *umuD*-like gene have been identified in gram-positive bacteria. It is possible that in these organisms UmuC proteins function alone. Two Y family DNA polymerases, YqjH (Pol Y1) and YqjW (Pol Y2) have been characterized in *Bacillus subtilis*. Both Pol Y1 and Pol Y2 are involved in UV-induced mutagenesis (Sung *et al*, 2003). The role and the requirements of Pol Y2 for DNA synthesis are similar to that of DNA pol V in *E. coli* (Duigou *et al*, 2004). Therefore Pol Y1 and Pol Y2 of *B. subtilis* can be functional homologues of *E. coli* DNA pol IV and DNA pol V, respectively. Mutagenesis by both of these DNA polymerases is dependent on DNA pol I, the polymerase participating in Okazaki fragment synthesis. The question remains whether these polymerases are capable of DNA synthesis by themselves or they act mainly as accessory factors and change DNA pol I specificity (Duigou *et al*, 2005).

In *Staphylococcus aureus* the Y family DNA polymerase SACOL1400 is induced under conditions of DNA damage and it confers UV resistance and mutability (Cirz *et al*, 2007). The gene for the Y family polymerase *umuC* is also present in *Streptococcus spp.*, a bacterial species lacking *lexA* and a classical SOS response (Cirz *et al*, 2006a). A UmuC homologue encoded in a damage-inducible mutagenesis cassette is present in several mobile elements of *Streptococcus* and *Lactococcus* species (Munoz-Najar & Vijayakumar, 1999; O'Driscoll *et al*, 2004; Varhimo *et al*, 2007). *umuC* was found to lead to higher frequency of mutations after UV irradiation in *S. uberis* (Varhimo *et al*, 2007).

4.2.2. DnaE proteins associated with UV mutagenesis

In the previous section I described *E. coli* DNA polymerase V and its orthologs in several bacterial species. *umuDC* orthologs are absent from most bacteria. The bacteria lacking genes encoding DNA polymerase V possess an operon composed of *lexA-imuA-imuB-dnaE2* genes. The operon is damage-inducible and under the control of a self-encoded LexA, so called LexA2. In several species this operon is involved in TLS and it likely functionally replaces *umuDC* in damage-inducible mutagenesis. In the operon, *dnaE2* encodes a second copy of the catalytic subunit of Pol III, a C family DNA polymerase responsible for chromosomal replication. Loss of DnaE2 does not lead to any growth defects; indicating that this polymerase is not essential for chromosomal replication (Boshoff *et al*, 2003; Galhardo *et al*, 2005). According to sequence alignment and its predicted structure ImuB resembles Y family DNA polymerases. Comparison with other Y family polymerases indicate that ImuB is not a polymerase as it lacks essential amino acids in its active site (Koorits *et al*, 2007). Currently it is believed, that ImuB participates in regulating polymerase traffic in replication fork. ImuA has an unknown function. This so called 'mutagenesis cassette' is widely distributed among proteobacteria, and exists in various different configurations. In some species LexA is not encoded within the operon; in these cases the operon is under negative control of LexA participating in the general SOS system. Often the number of copies of *imuB* and *dnaE2* varies in mutagenesis cassette (Erill *et al*, 2007). The operon and its role in mutagenesis, including UV-induced mutagenesis, has been described in some cases. In *Caulobacter crescentus* this operon is responsible for most UV-induced mutations (Galhardo *et al*, 2005). It has been suggested that *imuA*, *imuB* and *dnaE2* products cooperate in TLS process as no increase in UV-induced mutations was observed if either one was missing. The spectrum of mutations caused by UV light in wild-type, *dnaE2* and *imuB* strains has been determined. *dnaE2*$^{-}$ and *imuB*$^{-}$ strains show a similar increase in G:C→A:T transitions in the *rpoB* gene compared to the *dnaE2*$^{+}$*imuB*$^{+}$ strain, further suggesting a coupled role of these enzymes. Similar results have been obtained in *Deinococcus deserti*, where UV-mutable phenotype is dependent on simultaneous presence of DnaE2 and a second DNA polymerase, ImuY (B-family polymerase) encoded in the mutagenesis cassette (Dulermo *et al*, 2009). DnaE2 encoded in the mutagenesis cassette has also been characterized in *Mycobacterium tuberculosis* (Boshoff *et al*, 2003; Erill *et al*, 2006b). In this organism *dnaE2* was shown to be strongly upregulated in response to DNA damage, as well as to be required for UV resistance and UV-induced mutagenesis. A DnaE2-dependent increase in double CC→TT transitions (ACC CAC → ACT TAC) was observed in the *rpoB* sequence of UV-induced Rif^{R} mutants, indicating that DnaE2 is error-prone in synthesis across both of the nucleotides composing a pyrimidine dimer (Boshoff *et al*, 2003). In this study role of DnaE2 was analyzed independently from ImuA or ImuB. Therefore it is unclear, whether the DnaE2 needs the assistance of these proteins for TLS.

A homologue of the replicative DNA polymerases (C family) has been found in several bacteria. It has been estimated that ~50% of bacterial genomes carry at least two copies of *dnaE* or contain *dnaE* and *polC* (Le Chatelier *et al*, 2004). The second replicative DNA polymerase subunit can also exist independently of the mutagenesis cassette and in some species, for instance in *Streptococcus pyogenes*, *Enterococcus faecalis, Staphylococcus aureus*, *Bacillus subtilis* and related Gram-positive bacteria Pol C and DnaE are both essential for chromosome replication. In these species Pol C polymerase synthesizes the leading strand and DnaE polymerase the lagging strand (Dervyn *et al*, 2001). The role of these polymerases in UV mutagenesis has been investigated in *B. subtilis*. As the lagging strand synthesis polymerase DnaE is devoid of proofreading activity and is induced by the SOS response, it was proposed to be the error-prone polymerase needed for TLS. Surprisingly, the experiments revealed that both of the polymerases are essential for UV-induced mutagenesis in *B. subtilis* (Dervyn *et al*, 2001; Le Chatelier *et al*, 2004). The ability of DnaE to synthesize across different DNA lesions has also been assessed *in vitro*. The enzyme is not able to bypass either a benzo-(*a*)-pyrene adducted guanine (G-BaP) or a T-T 6-4PP dimer. As it can bypass an AAF adduct, a lesion that completely blocks progression of the *E. coli* pol III α subunit, quite efficiently, this polymerase was suggested to have a more relaxed active site compared to pol III α. *In vivo*, the replication fidelities of DnaE and PolC are comparable, indicating that although DnaE is induced approximately threefold upon DNA damage response, it is an error-free DNA polymerase (Le Chatelier *et al*, 2004). *S. pyogenes* DnaE and Pol C were also characterized *in vitro* by their ability to synthesize across different DNA lesions. It was found that DnaE can carry out TLS and extend the primer incorporating random nucleotides. This indicates that at least *in vitro* DnaE from *S. pyogenes* is a highly error-prone enzyme (Bruck *et al*, 2003). The lack of further data does not allow to judge whether DnaE proteins of *B. subtilis* and *S. pyogenes* are different in their fidelity when performing DNA synthesis *in vivo*. Also, the differences in *in vitro* bypass of the lesions may result from different lesions used as substrates in these two different studies. The *in vitro* data suggests that DnaE proteins of *B. subtilis* and *S. pyogenes* are capable of TLS. This observation does not necessarily mean that these polymerases are erroneous *in vivo*.

II STRESS-INDUCED MUTAGENESIS IN BACTERIA

1. Habitats of bacteria, life cycle and diversity

Bacteria are living in almost all kinds of environments one can imagine. In addition to ln soil, water, phyllosphere and enteric habitats certain species of bacteria are able to live in conditions of high osmolarity, extreme temperature and high pressure. Some species can even resist high radiation doses. Inhabiting various habitats is possibly achieved due to extreme ability of bacteria to adapt to changing environment. When new conditions are encountered, physiological adaptation occurs mediated by reprogramming of global genome activity and tuning of metabolic pathways to adjust to current conditions. In addition to physiological changes a transient increase in genetic variability occurs. The increased variability allows selecting for the bacteria that are most fit under current conditions. Genetic variability is the consequence of mutational processes: errors in replication, diminished repair activities, chromosome rearrangements and induction of transposable elements. The occurrence of all these different events increases under non-favorable growth conditions (Tsui *et al*, 1997; Ilves *et al*, 2001; Bjedov *et al*, 2003). This process of genetic adaptation caused by increased accumulation of mutations in bacteria in non-favorable conditions is referred to as stationary-phase mutagenesis or adaptive mutagenesis (Foster, 1999).

In this part of my thesis I will describe current understanding of the life of bacteria in a natural environment. I will focus on adaptation of bacteria to stress and changing growth conditions, with main emphasis on processes responsible for the increase of genetic variability.

1.1. Bacterial life cycle

Bacteria have been mostly studied in cultures where nutrients are not limiting and they are exponentially growing. In nature similar conditions possibly never occur and therefore one could question the relevance of such studies. In the majority of natural environments bacteria spend most of their existence under conditions of starvation. For instance, in aquatic environments the amount of nutrients is generally too low to provide growth of microorganisms. The amount of nutrients in the water is expressed as total amount of dissolved organic carbon (fatty acids, carbohydrates, proteins etc.) resulting from decomposition of organic matter such as plants and animals in water (Docherty *et al*, 2006). The concentration of organic dissolved carbon in seawater is typically 50 μM (Finkel, 2006), which is very low compared to 11 mM glucose (0,2 % glucose solution) usually added to defined minimal medium. The concentration of organic material in soil, which has much more organic matter compared to

aquatic environment, is still not sufficient to provide constant energy supply for bacteria (Morita, 1993). In terrestrial environments bacteria are subject to large variations in nutrient availability, being dependent on activities of other organisms, water and air flows. Undoubtedly, also in nature there are conditions where nutrients are abundant. For instance, mammalian gut is a good example of a protected environment constantly supplied with nutrients. Some species thrive well in this environment, but as it is inhabited by hundreds of bacterial species, in general the competition for nutrients is harsh and most of gut bacteria are still starving (Finkel, 2006). Thus even considering the existence of such nutrient-richer niches it seems that bacteria in their natural habitat spend most of their existence in non-favorable conditions, mostly due to starvation. Often bacterial life is referred to as 'feast or famine' (Kolter *et al*, 1993). The model suggests that bacteria consume readily all available, rapidly metabolizable nutrients in their environment. After the nutrients are exhausted, bacteria persist under starvation conditions. Life without additional external nutrients can last long periods of time. There is evidence of bacteria that have survived in stab cultures (without addition of nutrients) for more than forty years (Finkel *et al*, 2000).

Most of our knowledge concerning bacterial life in nature is not direct but rather extrapolated from studies on bacteria that have been kept for long periods in starvation in laboratory conditions. Patch and biofilm cultures incubated for long periods of time i.e. for weeks or for months have been used to characterize the processes occurring in bacterial cultures that have been subjected to long-term nutrient deprivation (Zambrano *et al*, 1993; Kraigsley & Finkel, 2009).

The bacterial life cycle under laboratory conditions is usually described by three phases: lag phase, exponential or logarithmic phase and stationary phase. Upon very long incubation without the addition of nutrients two additional phases have been described (Finkel *et al*, 2000). These are the death phase and the extended or long-term stationary-phase. I will next give a short overview of the nature of these phases (Figure 8), drawing most attention to the phases that are likely to prevail in natural habitats of bacteria: death phase and long-term stationary-phase. The molecular switches that lead to the transition of bacteria from one phase to another will not be discussed in this context.

When bacteria in a dormant state encounter nutrients this does not result in immediate growth. Initially bacteria adapt to the new conditions by synthesizing enzymes essential for metabolizing freshly encountered nutrients and proteins important for cell division. This period of preparations is referred to as the 'lag phase' and in laboratory conditions this phase occurs after inoculation of bacteria into a fresh medium. The length of the lag phase varies among different bacterial species as well as among bacteria from the same species, being dependent mostly on the amount of inoculated bacteria and their condition prior to encountering fresh nutrients. Gradually bacteria start dividing and soon a constant rate of cell division is achieved. This marks the end of the lag phase. The next period when the bacterial population grows exponentially is known as 'log phase'. During this phase the population expands and obtains most of its

biomass. The rate of bacterial cell division in this period is balanced, with the length of the generation time being dependent on the composition of the growth medium and other growth conditions. Exponential growth of the population may become limited due to many reasons: (1) after the nutrients have been exhausted; (2) by accumulation of metabolites or end products that inhibit growth or (3) by the exhaustion of available space. A population in which cells have slowed down their division rate enters the stationary-phase. Whereas the doubling time of *E. coli* is less than 30 minutes when growing exponentially in LB medium, after three days of incubation in the same medium the doubling time can be as long as 16–24 hours (Finkel *et al*, 2000). In stationary-phase the number of viable cells (colony forming units, CFU) in the population does not change. It is thought that the population size remains unchanged because the rate of cell growth and cell death are balanced. However, there are also studies showing that most of the bacteria in stationary-phase do not divide (Finkel *et al*, 2000; Roostalu *et al*, 2008). For instance, using flow cytometry cell division is detectable in a subpopulation that comprises less than 1% of the cells in the culture (Roostalu *et al*, 2008).

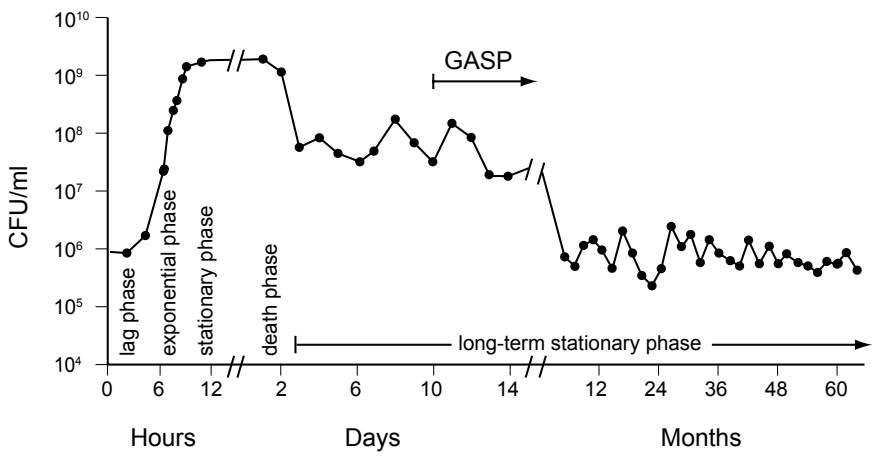


Figure 8. The five phases of bacterial life cycle as defined for growth under laboratory conditions. When bacteria are inoculated into fresh growth medium, there is an initial lag period followed by exponential-phase growth. After remaining at high density for 2 or 3 days (stationary-phase), cells enter death phase. After that ~99% of the cells die, while the survivors can be maintained under long-term stationary-phase culture conditions for months or even years. The arrow indicates the time point after which the cells expressing growth advantage in stationary-phase (GASP) phenotype are observed in *E. coli*. Adapted from Finkel, (2006).

Even without bacteria dividing, stationary-phase is not a quiescent phase. Many bacterial species produce secondary metabolites, e.g. antibiotics in stationary-

phase. Spore-forming bacteria in this phase induce processes needed for sporulation and non-sporulating bacteria undergo changes in gene expression that increase their resistance to stress. For instance, *E. coli* and also other species such as *Legionella pneumophila*, *Erwinia carotovora*, *Ralstonia solanacearum* and several *Pseudomonas* species, induce the so called general stress response (Flavier *et al*, 1998; Lange & Hengge-Aronis, 1991; Suh *et al*, 1999; Andersson *et al*, 1999; Bachman & Swanson, 2001; Miller *et al*, 2001). This phenomenon, leading to expression of genes encoding proteins to cope with endogenous damage and important for virulence, will be described later in a section "General stress response".

Regardless of environmental conditions, cells eventually loose viability. This marks the transition of the bacterial population to the next phase, death phase. As reflected by the name of this phase, during that period approximately 99 % of the cells die. In *E. coli* in LB medium, the death phase usually occurs three days after the inoculation. However, it varies depending on growth conditions and bacterial species. The mechanism that triggers transition of the cells from stationary-phase to death phase is not known. Two main hypotheses exist. The first one suggests that death of most bacteria in a population is a stochastic event triggered by endogenous damage and reduced repair due to limited energy supplies (Finkel, 2006). After a certain time of growth nutrients from the medium are consumed and cells lack the energy to carry out repair and maintenance functions i.e. cope with oxidatively damaged proteins and nucleotides (Nyström, 2003). Excessive amount of different types of damage causes death of most cells. As cells die, nutrients are released into the medium and the surviving fraction of population can catabolize their dead siblings. The second hypothesis considers bacterial death as a programmed event, so-called bacterial apoptosis. According to this model cells sense a high population density as well as nutrient limitation by a *quorum sensing* mechanism. This hypothesis supposes that bacteria have 'evolutionary knowledge', meaning that when a proportion of the population commits suicide the rest can survive and reproduce (Finkel, 2006). If nutrients become exhausted the population enters a so called death mode and commits an altruistic suicide. Some cells escape this program and upon availability of nutrients released from deceased siblings are capable of exit from 'apoptosis' and thereby persist. At this moment there is not enough knowledge to validate either of these hypotheses. It is clear that the endogenous damage (oxidized lipids and proteins and oxidatively damaged DNA) encountered by stationary-phase cells is one of the main reasons for their declined viability (Nyström, 2002). A short overview of types of oxidative damage threatening bacterial cells under stationary-phase conditions and the mechanisms by which bacteria protect themselves is presented in the next sections of this thesis.

After the death phase the cells that have remained viable can be incubated for long periods without adding any nutrients (Finkel & Kolter, 1999). These cultures can be maintained without any further loss in viability for more than five years at a density of approximately 10^6 colony forming units (CFU) per

milliliter (Finkel, 2006). This 'ever-lasting' period is known as long-term stationary-phase. As in the early stationary-phase, it is thought that in this period bacterial division and death are balanced (Figure 8). What actually occurs in this phase is yet unknown. There is evidence that the population turn-over is slow in these conditions, but also that only a small fraction of the population is dividing and the rest will remain viable but does not divide (Finkel *et al*, 2000; Roostalu *et al*, 2008).

1.2. Endogenous oxidative damage in starving bacteria

One of the main challenges for stationary-phase bacteria is coping with accumulating oxidative damage caused by reactive oxygen species (ROS: superoxide anions ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$)). Continuous oxidative stress is the reason why stationary-phase cells gradually lose their viability, despite of increased synthesis of antioxidant enzymes: superoxide dismutases and catalases (Hengge-Aronis, 2000). H_2O_2 and $\bullet O_2^-$ are generated as a byproducts of normal aerobic metabolism during respiration by the auto-oxidation of the respiratory dehydrogenases (Messner & Imlay, 2002). $\bullet OH$ is a secondary ROS, formed by electron transfer from ferrous iron (Fe^{2+}) to H_2O_2 (Figure 9) (Imlay, 2008). Due to its reactive nature ROS spontaneously damages all macromolecules: proteins, lipids, carbo-hydrates and nucleic acids (Demple & Harrison, 1994). Oxidative damage is also generated in fast-growing bacteria but in this case frequent cell division prevents accumulation of impaired macromolecules which are either repaired, diluted among daughter cells or degraded. Of all the macromolecules, oxidation of proteins has the largest impact on viability of bacteria upon encountering ROS (Nyström, 2002). Both primary ROS, $\bullet O_2^-$ and H_2O_2, damage proteins by oxidation of iron-sulfur clusters (4Fe-4S) and releasing catalytic iron to cytoplasm leaving affected enzymes inactive (Figure 9) (Flint *et al*, 1993). H_2O_2 also causes protein carbonylation *via* a metal-catalyzed oxidation reaction. Carbonylation of proteins is irreversible and this modification generally leads to inactive protein. Whereas moderately carbonylated proteins are degraded by the proteasome, heavily carbonylated proteins tend to form high-molecular-weight aggregates that are resistant to degradation and accumulate as damaged or unfolded proteins. Such aggregates of carbonylated proteins can inhibit proteasome activity and lead to further accumulation of damaged proteins (Dalle-Donne *et al*, 2006). Carbonylation is mediated by hydroxyl radicals that are produced from H_2O_2 in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + OH^- + H^+ \rightarrow Fe^{3+} + OH\bullet + OH^-$). While the Fenton reaction has been linked to protein carbonylation and also to membrane peroxidation, its major impact is likely on the DNA as even a single lesion induced in DNA is potentially mutagenic or lethal (Imlay, 2003). $\bullet OH$ produced in this reaction is the most reactive ROS. As it is extremely reactive, it damages mostly the macromolecules in the

immediate vicinity of the site of its production from H_2O_2 in the presence of free iron (Figure 9). Free iron in the cell is thought to be associated with metabolites and the surfaces of biomolecules, as well as with DNA which is therefore extremely vulnerable to oxidative damage (Rai *et al*, 2001). •OH damages sugar as well as base moieties in DNA producing more than 80 different types of lesions (Bjelland & Seeberg, 2003). The most common oxidative lesions are DNA strand breaks and oxidized guanine 7,8-dihydro-8-oxoguanine (8-oxoG). 8-oxoG and oxidized adenine 2-hydroxyadenine (2-OH-A) have been suggested to contribute directly to stationary-phase mutagenesis (Bridges, 1996; Saumaa *et al*, 2007). 8-oxoG pairs equally well with adenine and cytosine and so gives rise to G→T transversions. Analogously, 2-oxoA also pairs well with guanine leading to A→C transversions (Kamiya & Kasai, 2000; Brieba *et al*, 2004; Hsu *et al*, 2004).

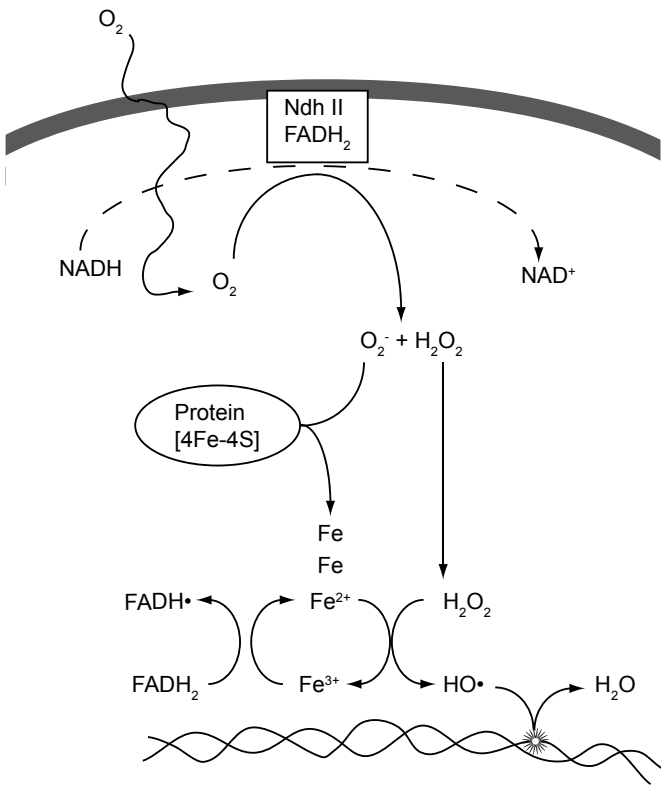


Figure 9. A typical series of reactions that generate oxidative DNA damage. Intracellular $•O_2^-$ and H_2O_2 are formed by auto-oxidation of flavoproteins. $•O_2^-$ oxidizes 4Fe-4S of proteins and releases iron into the cytosol. The iron catalyzes electron transfer from adventitious reductants to H_2O_2, thereby generating the hydroxyl radical that attacks the DNA. Adapted from Imlay, (2003).

1.3. General stress response

Some bacteria have evolved a stress response mechanism to deal with nutrient limitation and potential damage. This response known as general stress response is induced in cells confronted with various types of stresses including starvation. The aim of the response is not to provide the means for the repair but rather to tolerate stress caused by for instance carbon starvation, desiccation and oxidative damage and to secure the survival of bacteria in non-favorable conditions. The general stress response has been best studied in *E. coli* and therefore only the data available for this organism will be discussed. The general stress response in *E. coli* comprises a network of about 140 different genes regulated directly or indirectly by the stationary-phase sigma factor σ^s (σ^{38}, RpoS) encoded by *rpoS* (Weber *et al*, 2005). σ factors facilitate transcription initiation by enabling specific binding of RNA polymerase to promoters and allowing expression of certain sets of genes. Expression of the σ^s regulon is essential for survival of non-sporulating bacteria during long-term starvation and for coping with oxidative stress, high temperature, high osmolarity and acidic pH (Lange & Hengge-Aronis, 1991; McCann *et al*, 1991; Lee *et al*, 1995). The steady state of σ^s depends on the stress encountered and therefore induction of the σ^s regulon is not necessarily the same under different stress conditions (Mandel & Silhavy, 2005). Upon entry into the stationary-phase σ^s expression is triggered by the exhaustion of an essential nutrient, for instance carbon. Adaptation to starvation lasts approximately four hours. During that time the stress response genes are induced in a temporal manner and multiple stress resistance gradually develops (Hengge-Aronis, 2000). It is plausible that the conditions where *rpoS* and the genes in its network are induced, prevail in nature.

As mentioned above, σ^s is a subunit of RNA polymerase. Different sigma factors are expressed and incorporated into RNAP holoenzyme in response to different environmental stimuli to tune gene expression to newly emerging needs. σ^s is hardly present in growing cells, but is expressed in stationary-phase or in cells facing other stress (Lange & Hengge-Aronis, 1991; Jishage *et al*, 1996). In its amino acid sequence σ^s is similar to the 'housekeeping' sigma factor σ^{70} (RpoD) and it also recognizes similar promoter sequences as σ^{70}. Due to this shared quality σ^s has been proposed to take over the 'housekeeping' role of σ^{70} in stationary-phase cells (Typas *et al*, 2007). Despite recognition of similar promoter sequences these two sigma factors also facilitate the control of expression of different sets of genes (Gaal *et al*, 2001). Expression of these other, RpoS-specific genes is achieved in conjunction with additional regulatory factors, such as H-NS, Lrp, CRP, IHF or Fis and by minor deviations from the σ^{70} promoter consensus sequence that does not allow transcription initiation by RNAP containing σ^{70} (Hengge-Aronis, 2002).

It goes beyond the scope of this thesis to discuss here all the genes and pathways regulated by σ^s. Thus I will focus on those most relevant for my

thesis: the genes and pathways important for stationary-phase survival and in creation of genetic variability in bacteria under stress conditions. In the previous section I discussed the role of oxidative damage as one of the main causes of cell death. RpoS controls the expression of several genes involved in reducing oxidative stress and detoxification of reactive oxygen species. The catalases HPI and HPII (encoded by *katG* and *katE*) that detoxify H_2O_2 as well as periplasmic superoxide dismutase (encoded by *sodC*) scavenging superoxide are regulated by σ^s (Ivanova *et al*, 1994; Gort *et al*, 1999). Also, glutathione reductase (encoded by *gor*) which is important for detoxifying oxidative damage and Dps which is involved in DNA condensation are induced during general stress response (Becker-Hapak & Eisenstark, 1995; Grant *et al*, 1998). In addition to its DNA packing properties which by itself may protect DNA from being damaged, Dps protects DNA from oxidative damage by preventing formation of hydroxyl radicals *via* the Fenton reaction in the direct vicinity of DNA (see the previous section). Dps fulfills this function by binding free iron to its ferritin-like structure and making it unavailable for reacting with H_2O_2 (Grant *et al*, 1998). In addition to genes involved in stress tolerance, RpoS is known to control several mutagenic pathways. For instance it upregulates DNA pol IV (a DNA polymerase connected to stationary-phase mutagenesis) expression, and downregulates mismatch repair in stationary-phase cells, thereby increasing the frequency of replication errors (Tsui *et al*, 1997; Layton & Foster, 2003). The role of σ^s in stationary-phase mutagenesis will be discussed in the next section of this thesis.

1.4. Growth advantage in stationary-phase

Bacterial populations confronted with various types of stress including starvation, are dynamic. Each population consists of different subpopulations of cells that are dividing at different frequencies and cells that do not divide at all. Subpopulations in the same culture are in a different physiological state, but may also carry different mutations in their genomic sequence. A Subpopulation dividing faster than other in a certain moment will transiently exceed its number of viable cells relative to others. Thereafter its division slows down and another subpopulation emerges that can exceed its number. This type of 'waves' are common in bacterial cultures. Population dynamics and the occurrence of mutations that confer fitness in certain growth conditions can be analyzed in so-called *growth advantage in stationary phase* (GASP) experiment (Zambrano *et al*, 1993). This assay allows estimating fitness of one or the other population under given growth conditions. A population that exhibits the so-called GASP phenotype has accumulated mutations that enable 'better' growth under the conditions where it was selected. When conditions change, the mutant looses its fitness and the population is taken over by a 'new' mutant more fit under the changed conditions. The GASP phenotype is caused by genetic alterations, not by physiological changes. In *E. coli* this phenotype is stably inherited and can

be transferred from GASP-expressing to non-expressing bacteria *via* transduction (Zambrano *et al*, 1993).

In a 'classical' GASP experiment two initially isogenic cultures marked with neutral selection markers are incubated for different periods of time (for instance one day *vs*. ten days) and then cells from the older culture are introduced as a minority (usually 1:1000 dilution [v:v] into the younger culture. The GASP phenotype is evident as the cells from the longer incubated population gradually take over the culture until no cells from the initial population are detected (Zambrano *et al*, 1993). The usual time period essential for accumulation of GASP-conferring mutations is ten days for *E. coli* grown in LB patch culture. A population incubated for less than ten days has no growth advantage and cannot compete out the cells from a younger population. During extended periods of growth new 'waves' of GASP mutants are constantly arising and taking over the population (Finkel & Kolter, 1999). Cells from 20-day-old cultures outcompete cells from 10-days-old cultures, 30-days-old populations are more fit than 20-days-old populations and so on. The GASP phenotype has been observed for cultures as old as 120 days outcompeting 90-days-old cells. The cells from the long-aged cultures (i.e. for 120 days old), however, are no longer able to take over fresh overnight cultures. This is possibly the consequence of a too big difference in overnight culture conditions and long-term culture conditions. As the old cultures have adapted to growth conditions emerging in long-term cultures they are not fit anymore for overnight culture conditions.

GASP has been characterized both in gram-negative (*E. coli, Salmonella typhimurium*, *Shigella dysenteriae*, *Enterobacter cloaceae*, *Pseudomonas putida, Pseudomonas aureofaciens*, *Brucella abortus*) and in gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus globigii* and *Mycobacterium smegmatis*) (Eberl *et al*, 1996; Waterman & Small, 1996; Finkel *et al*, 2000; Martínez-García *et al*, 2003; Silby *et al*, 2005). The GASP phenotype arises in a broad range of conditions: in rich media such as LB as well as in minimal media supplemented with glucose or amino acid mixtures, in liquid and semisolid media as well as on LB agar plates and stabs (Finkel *et al*, 2000). Most of the GASP experiments to date have been done with planktonic cultures, and only recently it was shown that similar competition occurs in biofilm cultures of *E. coli* (Kraigsley & Finkel, 2009). The apparent general nature of emergence of GASP mutants has led to the hypothesis that these mutants may also arise in natural conditions and that long-term cultures can be applied as model systems to study processes occurring in natural environments.

Several GASP-conferring mutations have been isolated to date in *E. coli* (under conditions of LB planktonic culture). The first and best characterized is an allele of RNA polymerase, the σ subunit *rpoS* (*rpoS819*) (Zambrano *et al*, 1993). σ^s and its role in general stress response are described above in the section "General stress response". *rpoS819* has a duplication near the 3´ end of the gene, resulting in a protein in which the last four amino acids are replaced by 39, yielding a protein with reduced affinity for DNA. In addition to the well

characterized *rpoS819*, additional *rpoS* alleles yielding a GASP phenotype have been identified in aged *E. coli* cultures. Some of these mutations affect protein stability, whereas the effect of others still needs to be determined. All *rpoS* alleles yielding the GASP phenotype identified so far exhibit a partial loss-of-function phenotype in activating transcription from genes in the σ^s regulon. Some of these *rpoS* mutations have also been characterized in *E. coli* natural isolates (Ivanova *et al*, 1992; Jishage & Ishihama, 1997). As an *rpoS*-deficient strain does not confer a GASP phenotype over a wild-type strain, there is possibly a strong selection for partial σ^s functionality (Zambrano *et al*, 1993). A benefit could result from the reduced expression of genes responsible for slowing down the cell growth in a σ^s-dependent manner in stationary-phase cultures, with still sufficient expression of genes linked to stress resistance and for survival of the bacterial population during long term starvation.

Not all GASP mutations have been mapped to the *rpoS* gene. Moreover, the partial loss of function *rpoS* alleles confer the GASP phenotype only under the conditions under which they were selected i.e. in aerobically grown LB cultures (Farrell & Finkel, 2003). This observation suggests that some other mutations may be advantageous under different conditions (Finkel *et al*, 2000). Other GASP-conferring mutations and 'new GASP wave' mutations have been mapped to three regions on the *E. coli* chromosome. These loci were named *sgaA*, *sgaB* and *sgaC* for stationary-phase growth advantage (Zinser & Kolter, 1999). Later, a mutation in *lrp* was found to cause the *sgaA* phenotype and mutations activating the *ybeJ-gltJKL* operon were reported as responsible for *sgaB* phenotype. The nature of *sgaC* is still unknown. The *lrp* and *ybeJ-gltJKL* cluster encode the leucine-responsive protein and a high-affinity aspartate and glutamate transporter, respectively (Zinser & Kolter, 2000; Zinser *et al*, 2003). Lrp is a transcription factor which is induced upon transition into stationary-phase. It regulates the biosynthesis of leucine and other branched-chain amino acids, isoleucine and valine. The GASP allele of lrp, Lrp-1141, has an in-frame deletion of three basepairs in the DNA binding motif. As an Lrp-deficient strain has a similar GASP-conferring phenotype, Lrp-1141 is considered a loss of function allele, yielding an Lrp mutant unable to regulate transcription (Zinser & Kolter, 2000). As Lrp is responsible for activating amino acid anabolism while repressing the catabolism, loss of Lrp function would facilitate a metabolic shift towards using available amino acids released from dead siblings (Calvo & Matthews, 1994; Zinser & Kolter, 2004). A mutation activating the *ybeJ-gltJKZ* operon has been mapped to the gene adjacent to the operon: *cstA*, encoding cytidine nucleoside hydrolase (Zinser *et al*, 2003). This mutation allows bacteria to grow faster on glutamate, asparagine, proline and aspartate as carbon sources (Zinser & Kolter, 1999).

It has been speculated that GASP mutants are able to grow and take over a population in stationary-phase because they are growing faster than the parental cells on nutrients that become available from dying cells in the culture (Finkel *et al*, 2000). Most of the nutrients released from dead cells are expected to be amino acids, as they account for over half of the dry weight of *E. coli*. In

agreement with this notion, the GASP-conferring mutations isolated so far, *rpoS*, *sgaA* (*ybeJ-gltJKZ*), *sgaB* (*lrp*) and *sgaC* all allow faster growth on a mixture of amino acids (CAA) and also on nutrients obtained from dying cells (Zinser & Kolter, 1999). It is possible that secondary GASP mutants have acquired mutations that allow enhanced catabolism of other cellular components e.g. complex carbohydrates and lipids derived from cell wall and membrane components. For instance, it has been shown that *E. coli* mutants unable to 'consume' DNA suffer a significant loss in fitness (Finkel & Kolter, 2001).

In addition to metabolic properties there are other criteria that a population has to meet to aquire the GASP phenotype. As the GASP phenotype is not a physiological adaptation to starvation stress, but is the result of acquired beneficial mutations the appearance of the GASP phenotype can be used as a test-system to study the occurrence of mutations in stationary-phase populations. In *E. coli*, all three SOS inducible DNA polymerases are essential for the emergence of the GASP phenotype (Yeiser *et al*, 2002). Likely, these polymerases contribute to accumulation of the necessary mutations by carrying out erroneous DNA replication.

2. Stationary-phase mutagenesis

Mutational processes have been studied in bacteria already for several decades. Most of the research has as yet been done on actively growing bacteria and only recently studies characterizing mutational processes in starving bacteria have received more attention. The main motivations behind the arising interest are that (1) the conditions of stress caused by starvation prevail in nature, (2) bacteria in long-term stationary-phase undergo changes in gene expression that also affect mutational processes e.g. changes caused by σ^s.

Most of the studies on stationary-phase mutagenesis to date have been carried out in *E. coli*, but the presence of the phenomenon has been shown in other bacteria as well (Foster, 1999). It has been noted that bacteria under selection give rise to mutations that allow them to start growing in given conditions (Cairns *et al*, 1988). In long-term incubated cultures the mutation frequency is higher than would be expected given the population size and known fidelity of replication. Long-term grown *E. coli* cultures exhibit enormous genetic diversity: missense mutations, amplifications and deletions cover 1–2% of the genome (Farrell & Finkel, 2003; Zinser *et al*, 2003; Skvortsov *et al*, 2007).

The mutation spectrum in long-term starved bacteria differs from that of growing bacteria (Foster & Trimarchi, 1994; Kasak *et al*, 1997; Prival & Cebula, 1992; Rosenberg *et al*, 1994; Tegova *et al*, 2004), suggesting that the mechanism of occurrence of spontaneous mutations in growing and starving cells is different. These stationary-phase mutations (also referred to as adaptive mutations) are random and not directed (Foster, 1997). Analysis of stationary-phase mutations and the mechanisms underlying the phenomenon suggests the

involvement of two pathways in the process: general stress response and SOS response. According to the common view stationary-phase mutagenesis is caused by (1) malfunctioning of the MMR system that is down regulated by the general stress response, (2) erroneous DNA replication due to the involvement of SOS response-induced error-prone DNA polymerases in overall replication or in translesion synthesis and (3) increased amount of DNA damage caused by accumulation of toxic secondary metabolites and oxidative damage in non-dividing cells. SOS induction occurs spontaneously in static bacterial populations (Taddei *et al*, 1995). This means that SOS-induced DNA polymerases, DNA pols II, IV and V are all present in stationary-phase cells to carry out low-fidelity DNA synthesis.

There are several different test-systems to monitor the occurrence of genetic alterations in bacterial populations. Most of these rely on the ability to use new nutrient sources (e.g. lactose or phenol), obtain antibiotic resistance (Rif^R, Sm^R), or give the ability to synthesize a certain amino acid (histidine, tryptophan) for strains auxotrophic for synthesis of the same amino acid. I will not discuss the nature of various test-systems in this thesis. A thorough description of these test-systems can be found in the following references: (1) on the widely used test-system based on lactose utilization, FC40 in Foster, (2000); (2) on phenol utilization as a sole carbon source, Phe^+ systems in Kasak *et al* (1997) and Tegova *et al* (2004); (3) on obtaining rifampicin resistance, Rif^R in Garibyan *et al* (2003) and Severinov *et al* (1993); (4) on obtaining streptomycin resistance, Sm^R in Torii *et al* (2003); (5) on histidine and tryptophan auxotrophy, His^+ and Trp^+ in Koch *et al* (1996) and Ohta *et al* (2002), respectively. All these systems allow detection of either certain mutations at a certain position of the gene or a limited spectrum of mutations in one gene.

Stationary-phase mutagenesis is often described in bacteria starving for carbon source. There are two main approaches employing different conditions used in studying stationary-phase mutagenesis: in aged colonies or in populations starved on solid media. These conditions are selected as to reflect the nature of tightly packed and structured bacterial communities found for example in the gut environment as well as in the aggregated structures of bacterial biofilms and microcolonies found in soil and water habitats. In the next section I will describe the main results obtained using these two approaches.

2.1. Mutagenesis in bacterial populations on solid media

Test-systems that enable monitoring mutations occurring under non-lethal selective conditions such as starvation for a carbon source or a certain amino acid are often carried out on the solid surface of an agar plate containing the substance which utilization becomes possible only after a certain mutation in the target gene. Using these test-systems the occurrence of specific mutations can be observed for weeks before the viability of the non-dividing cells on the

plate starts to decline. The most widely used assay system in *E. coli*, FC40, is based on utilization of lactose as a sole carbon source. The system uses a +1 frameshift in a *lacI-lacZ* fusion gene located on the conjugative F plasmid in a Lac^- strain (Cairns & Foster, 1991). The frameshift in the fusion gene reduces the beta-galactosidase activity to about 1% of normal levels.

When the Lac^- cells containing the test-system are plated onto lactose minimal medium Lac^+ revertant colonies emerge. Colonies observed on the second day after plating reflect the spontaneous mutations encountered in FC40 during the growth under non-selective conditions in the liquid culture. Lac^+ colonies observed after the second day reflect the processes that have occurred on minimal plates. Sequence analysis of *lacI-lacZ* has revealed differences in the mutation spectrum in early arising Lac^+ mutations and starvation-dependent Lac^+ mutations. While the spectrum of growth-dependent mutations is very heterogeneous, including -1 deletions (not at mononucleotide repeats), larger frameshift-reverting additions and deletions, stationary-phase-dependent mutations are mostly -1 frameshifts in mononucleotide repeats (Foster & Trimarchi, 1994; Rosenberg *et al*, 1994). After the first week of starvation, Lac^+ colonies with no mutation in *lacI-lacZ* are also detected, constituting up to 50% of the isolated Lac^+ colonies. In those *lacI-lacZ* is amplified to 20–100 tandem copies. As *lacI-lacZ* is leaky, sufficient enzyme activity can be achieved to allow growth on lactose by amplification of the region (Hastings *et al*, 2000; Powell & Wartell, 2001; Kugelberg *et al*, 2006). This Lac^+ phenotype is transient and after successive growth cycles in rich medium these bacteria exhibit a Lac^- phenotype (Powell & Wartell, 2001). Both of these processes, occurrence of -1 frameshift mutations and *lacI-lacZ* involve induction of the general stress response controlled by σ^s (Layton & Foster, 2003; Lombardo *et al*, 2004).

I will further discuss the genetic nature of the Lac^+ colonies that emerged due to -1 deletion in *lacI-lacZ* during starvation. Lac^+ cells that result from frameshift mutations have an overall elevated mutation rate (from now on referred to as hypermutators) and due to that also carry an increased number of secondary unselected mutations in other genomic regions (Torkelson *et al*, 1997; Rosche & Foster, 1999; Godoy *et al*, 2000; Gonzalez *et al*, 2008). Occurrence of -1 frameshift mutations is dependent on double-strand-break repair (DSBR) and a σ^s mediated switch in repair synthesis from high-fidelity to error-prone (Ponder *et al*, 2005). Thus the proteins participating in recombination (RecA, RecBC and RuvABC) are essential for the occurrence of Lac^+. On top of that, SOS-induction of the specialized DNA polymerase, DNA pol IV (DinB), is required for the promotion of -1 frameshift mutations during repair synthesis (Cairns & Foster, 1991; Harris *et al*, 1994; Harris *et al*, 1996; Foster *et al*, 1996; McKenzie *et al*, 2000; McKenzie *et al*, 2001; He *et al*, 2006; Galhardo *et al*, 2009). It is likely that stress-induced mutagenesis also requires the other SOS-inducible DNA polymerases DNA pol II and DNA pol V. As FC40 counts only frameshift mutations that are not promoted by these specialized DNA polymerases, their role cannot be assessed using this test-system. Although upregulation of DNA pol IV by the SOS response is

necessary, it is not sufficient for the occurrence of frameshift mutations leading to Lac^+. Also, over-expression of DNA pol IV does not lead to increased frequency of frameshift mutations. Hence the switch from high fidelity to error-prone DNA synthesis mediated by σ^s does not only encompass an increase in *dinB* expression by an additional twofold, but it also facilitates recruitment of DNA pol IV to repair synthesis in DSBR in an as yet unknown manner (Kim *et al*, 1997; Courcelle *et al*, 2001; Layton & Foster, 2003). Likely, the recruitment is not accomplished by σ^s itself, but some other, yet unknown σ^s-controlled factors (Ponder *et al*, 2005; Galhardo *et al*, 2009).

In summary, the emergence of starvation-related Lac^+ colonies is dependent on the simultaneous occurrence of three distinct processes: (1) induction of the SOS response, (2) presence of a double-strand break and its repair and (3) induction of the σ^s-dependent general stress response. It is considered that together these events lead to emergence of a subpopulation of bacteria with a transient hypermutable phenotype (Galhardo *et al*, 2009). As the presence of any double-strand DNA break results in SOS induction and DNA damage itself can induce the σ^s regulon, which is already induced in bacteria under stress conditions, simultaneous occurrence of these processes may not be a rare event in cells (Pennington & Rosenberg, 2007; Merrikh *et al*, 2009).

2.2. Mutagenesis in aging colonies

A stationary-phase-dependent increase in mutation frequency is well established for aged *E. coli* colonies (Taddei *et al*, 1995; Bjedov *et al*, 2003). Of approximately 800 different *E. coli* strains (isolated from different hosts, sediments, air and water habitats) 13% showed more than 100-fold increase in mutation frequency during the seven days of starvation, and 53% of total isolates exhibited tenfold increase in mutation frequency during the test period (Bjedov *et al*, 2003). In these experiments colonies were 'created' by spotting approximately 10^3 cells from overnight liquid culture onto a nitrocellulose filter on fresh plates containing rich medium. The colonies were suspended and plated onto selective plates containing rifampicin (Rif) on day one after their initiation to monitor the mutations occurring in non-aged colonies and on day seven to monitor the mutations in aged colonies. (Bjedov *et al*, 2003).

Two different pathways have been identified as responsible for stationary-phase mutagenesis in *E. coli* aging colonies: ROSE for "Resting Organisms in a Structured Environment" and MAC for "Mutagenesis in Aging Colonies" (Taddei *et al*, 1995; Bjedov *et al*, 2003). ROSE was described in an *E. coli* laboratory strain. The raise in mutation frequency in aged colonies is in this case dependent on SOS induction mediated by cyclic-AMP (cAMP). The amounts of cAMP increase in bacteria transiting to stationary-phase, and this molecule is proposed to act as a signal for declined energy resources (Taddei *et al*, 1995). Although dependent on the SOS response, the increased mutation frequency in ROSE is independent of the error-prone DNA polymerase DNA pol V.

Surprisingly, UvrB and DNA polymerase I, both participating in the nucleotide excision repair pathway, are essential for the mutagenic phenotype (Taddei *et al*, 1997).

MAC on the other hand is dependent on oxidative metabolism. Although colonies are nearly anaerobic during the first days of growth, after exhaustion of nutrients and slowing down metabolic activity oxygen penetrates deeper into the colonies, causing oxidative damage (Ben-Jacob *et al*, 1998). The strains which showed 100-fold increase in mutation frequency in aerobic conditions showed only 1.9-fold increase in frequency of Rif^R mutations when grown in anaerobic condition (Bjedov *et al*, 2003).

The molecular mechanisms underlying the MAC phenotype were studied using one natural *E. coli* isolate with increased frequency of stationary-phase-dependent mutations (over 100-fold increase in mutation frequency compared to mutation frequency on day one) as an example. The pathway leading to increased mutation frequency is controlled by σ^s and cAMP regulons, and is not dependent on LexA and induction of the SOS response. Despite being independent of the SOS response, RecA and DNA pol II are essential for MAC. Possibly these proteins are needed because of their recognition of DNA lesions (DNA breaks) and their error-prone processing. Moreover, DNA pol II is also involved in the oxidative damage response, possibly mediating translesion synthesis over oxidative lesions (Escarceller *et al*, 1994). In good agreement with earlier results on σ^s regulated genes (Tsui *et al*, 1997), downregulation of mismatch repair (MMR) components was found to occur in the cells of aged colonies. Overexpression of the MMR damage recognition protein MutS significantly decreased the occurrence of MAC (Bjedov *et al*, 2003). Downregulation of mismatch repair in aged colonies is considered most important for increase in mutation frequency as loss of MMR function elevates mutation rates 100–1000 fold (Friedberg *et al*, 2006).

In addition to the characterization of processes essential for MAC, Bjedov *et al* have carried out computer simulations to estimate the effects of mutators on genetic adaptation (Bjedov *et al*, 2003). It was concluded that a population with a tenfold increase in stress-induced mutator phenotype will adapt up to 15% faster than a non-mutator phenotype, whereas a population with a 100-fold increase in mutation frequency will adapt up to 38% faster upon encountering stress.

Experiments carried out so far clearly indicate that there is no single pathway for stationary-phase mutagenesis. Still one can find similarities in the processes responsible for MAC, ROSE and rise in frameshift mutations in FC40. All these characterized phenomena are dependent on availability of error-prone DNA synthesis, the process mainly related to DNA repair synthesis either in NER or DSBR pathway. Given the understanding that chromosome replication is diminished in stationary-phase, the repair synthesis may be the only replication occurring under these conditions. Therefore, repair may serve yet another purpose in bacteria: it may provide the means for genetic variability that the evolution can act on.

RESULTS AND DISCUSSION

Aims of the study

Bacterial life is often described as feast and famine, where periods of nutrient availability alternate with long-term starvation and resting in stationary-phase. Under conditions where growth of bacteria is hampered due to nutrient limitations bacteria have elevated mutation rate. This increase in mutagenesis is often suggested as the combined result of DNA synthesis carried out by damage-inducible error-prone DNA polymerases and malfunctioning of mismatch repair. However, the phenomenon of stationary-phase mutagenesis cannot be explained simply by reduced fidelity of replication process. In resting cells the amount of endogenous, mostly oxidative, damage is increased. The saturation of GO repair has also been shown to be involved in stationary-phase mutagenesis. Although avoidance of mutations is generally needed to maintain the integrity of the genome and reduce the lethal effects of mutagenesis, the increase in mutation frequency observed in starving bacterial populations is important in adaptation of bacteria to growth in a changing environment.

In addition to endogenous damage, bacteria are confronted with exogenous damage caused by different chemicals and types of radiations. Lesions induced in response to exposure to DNA-damaging agents or radiations can be repaired in different pathways or tolerated. It is well established that damage repair is error-free, whereas damage tolerance is generally mutagenic. Due to its effects on mutation frequency, damage tolerance is usually considered as ultimate option to survive and it is used only if there is no other possibility left. Although there are numerous data available on the effect of DNA damage repair and tolerance mechanisms on mutation frequencies in growing bacterial populations no data is available on the contribution of these systems to mutagenesis in stationary-phase populations under high level of endogenous damage. Here, we study how the pathways of repair and tolerance of UV-induced damage contribute to mutagenesis in resting bacteria. We also study the protection of UV-induced damage repair and tolerance systems on growing bacteria under conditions of UV exposure.

As a model system we use *Pseudomonas putida*. *P. putida* belongs to genus *Pseudomonas*, one of the most prominent groups of bacteria able to colonize various ecological niches including soil, plants and animals. *P. putida* is a non-pathogenic member of this genus. As pseudomonads live in a constantly changing environment, studying mutagenesis in this species provides insights into the processes that make these bacteria fit in adapting to new growth conditions.

Studying mutational processes and identifying types of mutation requires feasible test-systems. In the following section I will describe briefly the test-systems used in our studies

Test-systems for the study of mutational processes

In our experiments we use two different test-systems to study mutational processes in growing and starving populations of *P. putida*. To estimate spontaneous or damage-induced mutagenesis in growing bacteria we use the Rif^R assay (Figure 10). This assay permits detection of mutations that yield resistance to rifampicin and is widely employed for investigation of mutational processes in different bacterial species. Rifampicin binds to the RNA polymerase β subunit within the DNA/RNA channel and blocks further elongation of the RNA transcript as it reaches 2–3 nt in length (Campbell *et al*, 2001; Hartmann *et al*, 1967). Most of the rifampicin resistance (Rif^R) conferring mutations are mapped to residues in the β subunit that either make direct contacts with rifampicin or are located near the rifampicin binding pocket (Campbell *et al*, 2001). These mutations decrease the affinity of rifampicin for the enzyme making the enzyme insensitive to the antibiotic and allowing transcription elongation to occur (Artsimovitch *et al*, 2005). Almost all the mutations encountered using this test-system are base substitution mutations; in-frame deletions or insertions occur at a low frequency (Campbell *et al*, 2001). The mutations conferring Rif^R to *P. putida* are described in Figure 10 (Jatsenko *et al*, 2009). As rifampicin is a bactericide it only allows detection of mutations that have occurred prior to plating the cells onto rifampicin-containing plates. After overnight incubation of *P. putida* cells on rifampicin containing plates less than 0.2% of cells survived (Jatsenko *et al*, 2009).

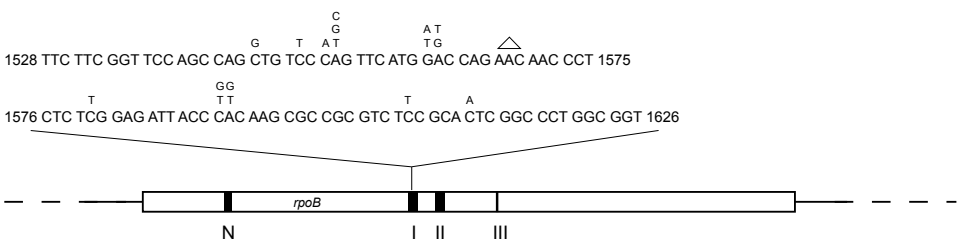


Figure 10. Mutations conferring the Rif^R phenotype to *P. putida*. The bar schematically represents the primary sequence of the β subunit (encoded by *rpoB*). Dark segments indicate the four clusters where Rif^R mutations have been identified in *E. coli*, denoted as the N-terminal cluster (N), and clusters I, II, and III (I, II, III), respectively (Campbell *et al*, 2001). In *P. putida* all Rif^R-conferring mutations map to cluster I. Therefore, only the sequence of this cluster is shown on the figure. Numbers at 5′ and 3′ of the sequence indicate positions of the nucleotides relative to the start codon ATG. Base substitutions that confer Rif^R in *P. putida* are indicated above the sequence. The open triangle designates the deletion of three nucleotides which also confer resistance to Rif (Jatsenko *et al*, 2009).

For studying stationary-phase mutagenesis we use the Phe^+ test-system. This system, based on utilization of phenol as a carbon source was developed by us (Tegova *et al*, 2004). The plasmid-encoded phenol monooxygenase gene *pheA* is interrupted either by a translation stop codon TAG or a +1 frameshift mutation (Figure 11). Bacteria plated onto minimal plates containing phenol as a sole carbon source start phenol utilization and form colonies only when a mutation occurs that reverts the stop codon or restores the correct reading frame. Bacteria can starve for long periods on phenol plates without loosing viability. With this system, mutational processes can be followed over time periods as long as two weeks. The Phe^+ test-system also allows detection of spontaneous mutations, albeit at low frequency. Phe^+ reversions that occur in cultures prior to spreading the cells on phenol plates result in colonies on the second day after plating. Mutants emerging on day three or later reflect the events occurring on phenol plates.

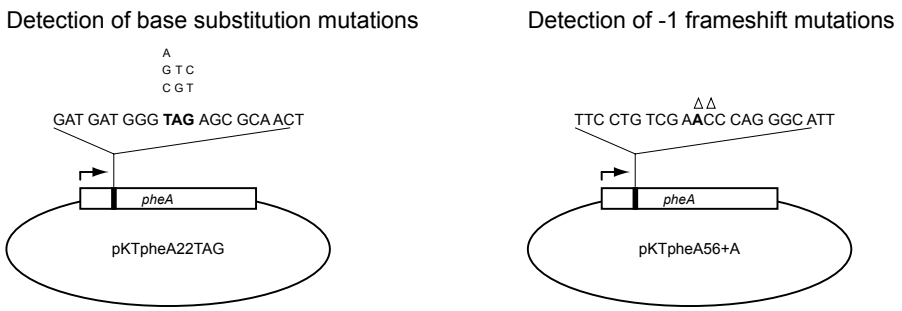


Figure 11. Phe^+ test-systems allowing detection of base substitution mutations and 1-bp deletions. Plasmids for the detection of base substitution mutations pKTpheA22TAG and 1-bp deletions pKTpheA56+A are indicated as ovals. The bar represents the phenol monooxygenase gene *pheA* under the control of a constitutively expressed promoter (indicated by an arrow). Introduced arrangements in original *pheA* gene (TAG or +A) are shown in bold in their sequence context. Nucleotides forming a codon in the unchanged *pheA* sequence are separated by space. Mutations that result in reversion to Phe^+ are indicated above the sequence. The open triangle indicates site at which Phe^+-conferring nucleotide deletion occurs (Tegova *et al*, 2004).

The results obtained using either Rif^R or Phe^+ test-system cannot be directly compared due to the differences in types of mutations that are detected, test-gene sequence context as well as the localization of test-genes on chromosome in case of Rif^R and on plasmid in case of Phe^+.

I MUTAGENIC POTENTIAL OF DNA DAMAGE REPAIR IN STATIONARY-PHASE POPULATIONS

UV-induced DNA damage can be repaired *via* different pathways. In the Literature Review chapter of this thesis I have described the systems participating in removal of CPDs or 6-4PPs as well as other UV-induced lesions in bacteria. However, no species encode all these described systems in its genome. Bacteria mostly rely on photolyases removing CPDs and nucleotide excision repair (NER) proteins removing a wider spectrum of lesions. *Pseudomonas putida* is no exception to that.

Our interest was to study the mutagenic potential of repair in resting cells under conditions where no DNA damage is introduced by UV or other exogenous factors. Bacterial photolyases are strictly limited to repair of CPDs and recognize no other type of DNA damage: therefore repair by these proteins would not be expected in the absence of UV radiation. NER, although characterized by its major role in repair of UV-induced damage, can recognize a large variety of structurally unrelated DNA alterations (Truglio *et al*, 2006a). It is surprising that if the recognition and removal of different substrates is compared in *in vitro* reaction conditions, CPD and 6-4PP adducts are relatively bad substrates for NER, suggesting that there may be other lesions in cells removed by NER in more efficient fashion. NER with its broad damage recognition spectrum is likely functional also in resting cells. Indeed, it has been shown that NER functions as a backup for DNA glycosylases in repair of oxidized or methylated bases, lesions which amount dramatically increases in stationary-phase populations (Moller & Wallin, 1998; Grzesiuk *et al*, 2001). It has also been suggested that there is a co-operation of BER and NER in a way that apurinic (AP) sites can serve as substrates for NER (Lin & Sancar, 1989; Snowden *et al*, 1990; Asad *et al*, 2000).

The genes for NER proteins UvrA, UvrB and UvrC needed for DNA damage recognition and incision are present also in the genome of *P. putida*. Additionally, *P. putida* harbors a 2517-nt open reading frame PP3087 encoding another, putative UvrA (J. Craig Venter Institute, Comprehensive Microbial Resource). The amino acid sequence of this homologue has 44% identity with *P. putida* UvrA (PP0483 product) and it classifies as a class II UvrA protein (UvrA2 family protein) by sequence analysis. We named the corresponding gene *uvrA2*. UvrA2 proteins, also known as DrrC and SnorO in *Streptomyces* species, give resistance to DNA intercalating agents (Lomovskaya *et al*, 1996; Ylihonko *et al*, 1996). This process has been proposed to be carried out by UvrA2 proteins independent of UvrB and UvrC (Goosen & Moolenaar, 2008). Surprisingly, neither the role of class II UvrA proteins in NER nor the need for UvrB or UvrC in giving resistance to intercalating antibiotics has ever been assessed. Compared to UvrA, UvrA2 proteins lack one domain which function was long unknown. However, the recently published UvrA crystal structure revealed that this domain is needed for UvrA-UvrB interaction and it is therefore essential for UvrA proteins in carrying out the NER reaction

(Pakotiprapha *et al*, 2008). Although devoid of the UvrB interaction domain, UvrA2 proteins can transiently interact with UvrB (Timmins *et al*, 2009), suggesting that the interaction between these proteins can be mediated by some other domain.

1.1. UV-tolerance of nucleotide excision repair-deficient *P. putida* (Reference I)

In order to study the effect of NER on survival of *P. putida* in the presence of UV-induced DNA damage we examined the sensitivity of WT, *uvrA*-, *uvrB*- and *uvrC*-deficient strains to UV-C irradiation at various doses. To assess whether UvrA2 could participate in NER, we carried out parallel experiments using an *uvrA2*-deficient strain and an *uvrA2uvrA* double-deficient strain. The data presented in Figure 12 (Reference I, Figure 1) show that compared to WT all NER-deficient strains exhibit reduced tolerance to UV radiation. Interestingly, the lack of UvrA does not cause as strong UV sensitive phenotype as the absence of UvrB or UvrC. This indicates either that UvrB and UvrC recognize lesions and incise them without the assistance of UvrA, or that some other factor like UvrA2 may substitute for UvrA in the damage recognition process. Deficiency of UvrA2, however, does not cause any reduction in survival of bacteria upon exposure to UV radiation compared to WT. Though, in the *uvrA2uvrA* double-deficient strain, the surviving fraction of bacteria is significantly decreased in comparison with the *uvrA*-deficient strain, suggesting that UvrA2 does confer resistance to UV. The *uvrA2uvrA* double-deficient strain tolerates higher doses of UV than the mutants lacking either UvrB or UvrC. This indicates that even if UvrA2 partially complements UvrA in damage recognition process, UV-induced lesions can be removed by UvrB and UvrC alone.

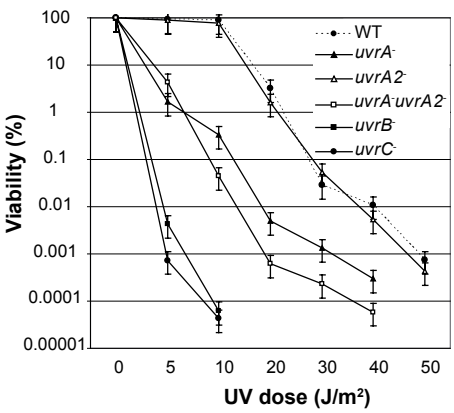


Figure 12. UV survival of *P. putida* wild-type strain and its NER-deficient derivatives at various UV doses. Data (percentage of survival) are expressed as the number of UV irradiated cells forming colonies as a fraction of the colonies formed by unirradiated cells and represent the median value of at least 60 independent measurements for each strain at each UV dose. Error bars represent 95% confidence level.

Our results indicate that in *P. putida* as in other organisms, NER is an important pathway in repair of UV-induced damage. UvrA2 participates in the process providing UV-tolerance to *P. putida*, but it likely has only a minor role in this process.

1.2. The role of UvrA2

UV-tolerance experiments show that deletion of *uvrA2* from an *uvrA*⁻ strain further reduces the viability of bacteria upon exposure to UV radiation. A similar effect has been described by Shen *et al* when studying the role of UvrA and UvrA2 in UV-tolerance in *Xanthomonas axonopodis* (Shen *et al*, 2007). In addition, the *uvrAuvrA2* double-deficient strain shows increased sensitivity compared to *uvrA*⁻ towards other DNA damaging agents such as methyl methanesulfonate (MMS) and mitomycin C (MMC) (Shen *et al*, 2007). Based on these results the authors suggest that UvrA2 is auxiliary to UvrA and assists UvrA in the DNA repair process. In *Deinococcus radiodurans*, in contrast, the lack of UvrA2 has no effect on UV-tolerance even in an *uvrA*-deficient background (Tanaka *et al*, 2005). Taking into account that *D. radiodurans* harbors multiple redundant repair systems for UV-induced damage repair, a modest effect of UvrA2 may simply have gone unnoticed in this organism.
The results of our UV-tolerance studies and data by Shen *et al*, 2007 indicated that UvrA2 may have a role in UV-induced damage repair. Based on these results it is difficult to say whether the role of UvrA2 is dependent on NER or not. In the study described in the following section we have investigated possible participation of UvrA2 in the NER pathway using two different approaches: (1) by carrying out *in vitro* DNA binding and damage incision assays; (2) by testing whether UvrA2 proteins mediated tolerance to DNA intercalating antibiotics is dependent on NER enzymes and whether UvrA can mediate the same process.

1.2.1. *In vitro* studies

For carrying out *in vitro* studies we purified the *P. putida* NER proteins. UvrA, UvrB and UvrC were purified according to previously published protocols (Visse *et al*, 1992; Moolenaar *et al*, 2001). UvrA2 was purified using the same protocol as UvrA. Protein purification and *in vitro* experiments were carried out in the laboratory of Dr Nora Goosen at Leiden University, The Netherlands. This work was supported by EMBO Short-Term Fellowship (ASTF 404-07).

Damage discrimination properties of UvrA2

We assessed the DNA binding and undamaged-damaged DNA discrimination properties of UvrA2 in comparison to UvrA using a filter binding assay (Malta *et al*, 2007). In this assay increasing amounts of UvrA2 or UvrA are incubated with a radioactively labeled 96 bp DNA substrate that is either undamaged or contains a centrally located fluorescein adduct as a damage. The reaction mixture is poured over the nitrocellulose filter and washed to retain only the DNA that is in complex with protein to membrane. As UvrA2 tends to aggregate in solution we cannot directly compare the amounts of UvrA and UvrA2 in our assays. According to atomic force microscopy (AFM) measurement, in the preparation of UvrA2 used in the assays 60% of the protein is aggregated while UvrA is non-aggregated (Wagner, personal communication). The results shown in this section are presented such as to give similar degrees of DNA binding to damaged DNA for both UvrA2 and UvrA. The protein concentrations as indicated in the figures correspond to the total amount of protein added to reactions. The aggregation-prone behavior of UvrA2 observed by us is confirmed in studies by Timmins *et al*, (2009). According to their observations, UvrA2 precipitates in the absence of cofactors, ATP or ADP. DNA binding experiments carried out by us without a cofactor in the reaction mixture showed no DNA binding by UvrA2 while binding of UvrA was only slightly affected in similar conditions (data not shown). Aggregation of UvrA2 in the absence of a cofactor would explain this result. The data presented in Figure 13A show that UvrA2 binds preferentially to damaged DNA in the presence of ATP. Its damage discrimination properties are even slightly better than those of UvrA (Figure 13B).

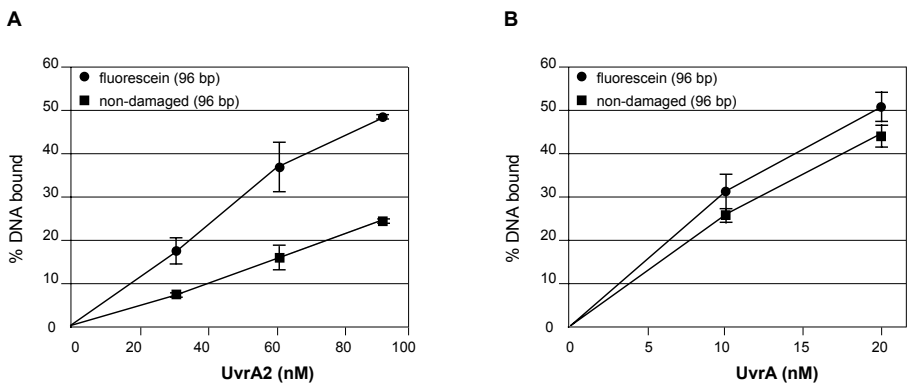
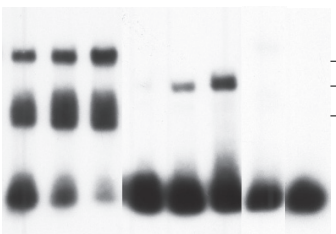


Figure 13. Binding of UvrA2 (**A**) and UvrA (**B**) to damaged and undamaged DNA substrates (96 bp). Damaged DNA substrate contains a fluorescein adduct in central position. DNA substrate concentration in experiments is 1 nM. Protein binding is expressed as the percentage of the input DNA on the filter, each sample is corrected for the amount of DNA retained on a filter in the absence of protein.

Participation of UvrA2 in nucleotide excision repair

In order to address the participation of UvrA2 in NER we studied whether the protein can substitute UvrA in preincision complex formation and damage incision assays. First we assessed UvrB-DNA preincision complex formation in the presence of either UvrA2 or UvrA. The UvrB preincision complex can be joined by UvrC, upon which incisions are carried out at both sides of the damage. For visualization of different protein complexes on DNA, we carried out gel retardation assays as described in Visse *et al* (1992). This assay relies on the formation of different protein:DNA complexes in the course of NER reaction which have different mobility in native gel. As damaged DNA we used 50 bp dsDNA with fluorescein at a central location. In the presence of UvrA and UvrB in the reaction mixture an UvrB:DNA complex with higher mobility than UvrA:DNA or UvrAB:DNA complexes is formed (Figure 14). UvrA:DNA and UvrAB:DNA complexes are indistinguishable in a native gel (Visse *et al*, 1992). When UvrA2 is used instead of UvrA in the assay, no complex with higher mobility (i.e. an UvrB:DNA complex) can be detected on the gel (Figure 14). UvrB:DNA complex formation and subsequent UvrC binding are essential for the NER reaction to occur. As in the presence of UvrA2 UvrB loading onto DNA cannot be detected, the protein likely cannot complement UvrA in NER.

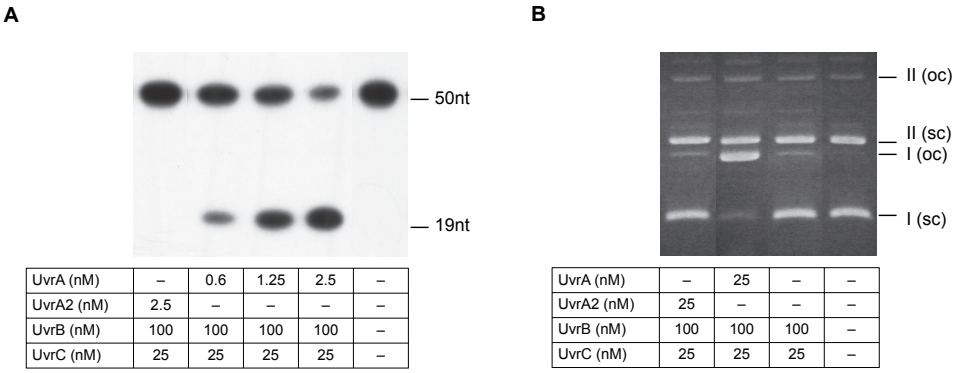


UvrA (nM)	2	4	8	-	-	-	4	-
UvrA2 (nM)	-	-	-	2	4	8	-	4
UvrB (nM)	100	100	100	100	100	100	100	100

Figure 14. Formation of UvrB:DNA preincision complex in the presence of UvrA2 and UvrA. Different protein-DNA complexes are visualized on native gel. – denotes non-damaged DNA substrate. DNA substrate concentration in experiments is 100 pM.

To verify that UvrA2 cannot participate in NER in a manner similar to UvrA we carried out an incision assay that estimates excision of the damage from the DNA substrate (Visse *et al*, 1991). As a substrate (1) 50 bp damaged DNA with radioactive label in the 5′ end of the damaged strand or (2) to more closely approach physiological conditions supercoiled plasmid can be used. When using linear DNA as a substrate incision results in a shorter 5′ labeled DNA

fragment (19 nt), which can be visualized on denaturing gel. When using supercoiled (sc) plasmid as a substrate, incision can be detected by appearance of a relaxed plasmid (oc) band on an agarose gel. In order to follow damage-specific incision a mixture of UV-irradiated (I) and non-irradiated (II) plasmids is used in the assay. Relaxation of only UV-irradiated plasmid should occur. When UvrA, UvrB and UvrC are used in this assay, damage-specific incision is detected on both substrates (Figure 15A and 15B). No incision is detected if UvrA2 is used in this experiment instead of UvrA (Figure 15A and 15B). The results obtained in incision assays confirm that UvrA2 is not capable of participating in the NER pathway in a ´classical´ way i.e. to lead to functional preincision complex formation and damage incision after damage recognition. The results do not exclude a role of UvrA2 in NER as secondary damage recognition protein, analogous to Mfd or ATL participating in recruiting NER to specific lesions – in these cases stalled RNA polymerases or O^6-alkylguanine, respectively (Selby & Sancar, 1993; Morita *et al*, 2008).



UvrA (nM)	–	0.6	1.25	2.5	–
UvrA2 (nM)	2.5	–	–	–	–
UvrB (nM)	100	100	100	100	–
UvrC (nM)	25	25	25	25	–

UvrA (nM)	–	25	–	–
UvrA2 (nM)	25	–	–	–
UvrB (nM)	100	100	100	–
UvrC (nM)	25	25	25	–

Figure 15. Incision of DNA in the presence of UvrA2, UvrB and UvrC or UvrA, UvrB and UvrC. **A.** Using linear 50 bp damaged DNA with radioactive label in the 5´ end of the damaged strand as a substrate. Incision results in 19 nt DNA fragment. Concentration of DNA substrate in reaction is 200 pM. **B.** Using mixture of UV-irradiated (300 J/m^2) plasmid (pUC18, 2686 bp) (I) and non-irradiated plasmid (pNP228, 4686 bp) (II) supercoiled (sc) plasmids as a substrate. 50 ng of each plasmid was used in the reactions. Incision results in relaxation of the plasmid (oc). – denotes proteins replaced by dilution buffer. Reactions are stopped after 15 minutes of incubation at 30°C.

1.2.2. *In vivo* studies using intercalating adducts as DNA damage

The UvrA2 proteins studied so far have a significant effect on survival of bacteria only when their role in resistance to the DNA intercalating antibiotics daunorubicin and nogalamycin is assessed. As streptomycetes and some other UvrA2 harboring species produce DNA intercalating agents to inhibit the growth of other bacteria, it has been proposed that UvrA2 proteins provide a

mechanism to protect their own cellular machinery from the cytotoxic action of these synthesized compounds (Lomovskaya *et al*, 1996).

We were interested in whether the resistance to intercalating antibiotics observed in *Streptomyces* species is an intrinsic property of UvrA2 proteins or whether this process is dependent on UvrB and can be carried out also *via* the NER pathway. Genome analysis of all sequenced bacterial species has revealed that some bacteria encode several (up to four in *Clostridium difficile*) UvrA2 proteins in their genome. This fact has led to the suggestion that different UvrA2 proteins have different specificities for DNA damaging agents (Goosen & Moolenaar, 2008). There is currently no evidence that *P. putida* produces DNA intercalating agents. Therefore we cannot speculate on what could be the specificity of the UvrA2 protein in this species. To assess whether UvrA2 and also NER target DNA intercalating agents we tested the growth of UvrA2 and NER-deficient strains on the plates containing daunorubicin or ethidium bromide, at concentrations of 7.5 and 125 μM, respectively. Experiments were carried out in the presence of membrane-permeabilizing agent citrate to allow efficient entrance of the antibiotics into the cell. Shortly, overnight-grown bacteria were diluted into fresh minimal medium supplemented with citrate. At cell density of A_{580}=0.6 serial dilutions were spotted onto minimal plates with and without antibiotics. Images were taken after incubation of plates at 30°C for 20 hours. All strains grew equally well on plates without antibiotics (data not shown).

Surprisingly, the UvrA2-deficient strain tolerates both tested antibiotics as well as the WT (Figures 16A and 16B). The ability of UvrA2 to increase tolerance of bacteria to intercalators is evident only in the *uvrA*$^-$ background, when a significant decline in viability is observed. The UvrA-deficient strain exhibited a slightly lower viability on ethidium bromide and daunorubicin plates than WT and UvrA2-deficient strain (Figures 16A and 16B). These results indicate that UvrA2 and UvrA both protect *P. putida* from these intercalating agents and that these proteins fully complement each other in this process.

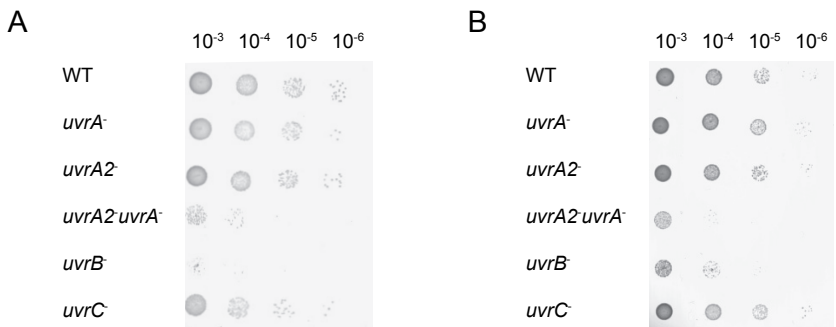


Figure 16. Sensitivity of *P. putida* UvrA2 and NER-deficient strains to intercalating agents. **A.** Daunorubicin **B.** Ethidium bromide.

Next, we asked whether the resistance to intercalating DNA damage is achieved in a UvrB- and UvrC-dependent manner. The UvrB-deficient strain is as sensitive to ethidium bromide and daunorubicin as the *uvrAuvrA2* double-deficient strain, suggesting involvement of UvrB in the pathway (Figures 16A and 16B). Deletion of UvrC did not have any affect on viability of bacteria exposed to daunorubicin or ethidium bromide, indicating that the moieties intercalated into DNA do not need to be excised from the DNA, but possibly can be flipped out by the action of UvrA2/UvrA and UvrB on DNA. Hence, we propose a model for UvrA2-UvrB or UvrA-UvrB-mediated resistance to intercalating agents (Figure 17) according to which UvrB and UvrA2/UvrA participate in different steps of the same pathway with UvrA2/UvrA likely recognizing the change in DNA conformation and recruiting UvrB for flipping out the adduct.

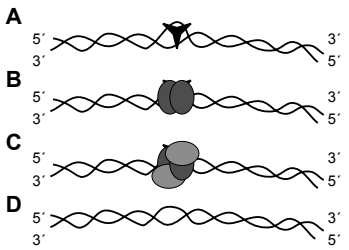


Figure 17. A proposed model of resistance to DNA-intercalating agents facilitated by UvrA or UvrA2 and UvrB. **A.** DNA intercalating agent binds to DNA **B.** UvrA and UvrA2 recognize distortions in DNA caused by intercalator binding **C.** UvrB is recruited to complex **D.** Intercalator is flipped out of the DNA during the damage search process.

Our results indicate that in addition to UvrA, UvrA2 can interact with UvrB in the process that gives resistance to intercalating agents. Although UvrA2 lacks the domain *via* which UvrA proteins interact with UvrB, transient interactions between UvrA2 and UvrB occur (Timmins *et al*, 2009). Although this interaction does not lead to formation of an UvrB preincision complex on DNA, it can be sufficient to facilitate damage localization by UvrB, during which the intercalators are likely removed from DNA. The finding that UvrA and UvrB participate in addition to UvrA2 in the pathway giving tolerance to intercalators is in good agreement with *in vitro* studies in which it has been shown that addition of intercalating antibiotics to incision assay mixture inhibits repair of covalent damage. This possibly occurs by recruitment of UvrA and UvrB to intercalator-undamaged DNA complexes (Selby & Sancar, 1991).

Our results indicate that although UvrA2 is incapable of substituting UvrA in the canonical NER reaction, the protein participates in a NER sub-pathway providing protection against intercalating DNA damage with efficiency similar to UvrA.

1.3. Role of nucleotide excision repair in mutagenesis (Reference I)

In order to investigate the role of NER in mutational process in the absence of exogenous damage we measured the frequency of Phe^+ revertants in growing and starving populations of WT and in strains deficient in different NER enzymes and UvrA2.

1.3.1. Role of nucleotide excision repair in mutagenesis in growing *P. putida*

In the Phe^+ test-system mutations occurring in growing cultures are reflected in the number of colonies emerging on phenol plates on the second day after plating. Surprisingly, the frequency of spontaneous Phe^+ mutations declines in all NER-deficient strains investigated (*uvrA*$^-$, *uvrB*$^-$, *uvrC*$^-$) as well as in *uvrA2*$^-$ and *uvrA2*$^-$*uvrA*$^-$ double-deficient strain. Although the number of viable cells plated per selective plate was approximately 1×10^9 both in case of the WT strain and its NER-deficient derivatives, the majority of plates with NER-deficient bacteria do not have any Phe^+ colonies on the second day. Hence, the mutation frequency is below the detection level in these strains, whereas that of the WT strain is 0.8×10^{-9} (Table 1; Reference I, Table 2).

To increase the sensitivity of our test-system we decided to repeat the experiment in a mismatch repair (MMR) deficient background. MMR is essential for repair of mismatches occurring during replication. In *MMR*$^-$ strains replication errors are left unrepaired and thus the mutation frequency elevates drastically. Deletion of the MMR enzyme MutS in *P. putida* results in approximately 60-fold increase in mutation frequency (Saumaa *et al*, 2006). As MMR has a preference for repair of certain mismatches (Joshi & Rao, 2001), mutations can be detected and observed without any effects of preferential repair in the absence of MMR. Results obtained for NER mutants in a MutS-deficient background differ drastically from that obtained in MutS proficient strains (Table 1; Reference I, Table 2). Whereas in the WT background all NER and UvrA2-deficient strains show a decline in mutation frequency, an increase of mutations is observed in the *uvrAmutS* double-deficient strain. The simultaneous absence of UvrA and MutS elevates the median frequency of the mutations about 11-fold compared to the MutS strain. These data imply that in addition to correcting exogenously damaged DNA, NER might back up MMR in repairing replication errors. On the other hand the absence of UvrB or UvrC does not elevate the mutation frequency in MutS-deficient background compared to *mutS*$^-$. Thus, NER in general plays no role in avoidance of spontaneous mutations in *P. putida*.

UvrA and UvrA2 have opposite effects on mutagenesis

To assess whether the elevated mutation rate in the *uvrA⁻mutS⁻* strain is caused by action of UvrA in the NER pathway or rather connected to its other suggested roles in recombination or replication, we tested the frequency of Phe^+ revertants in a *uvrAmutSuvrB* triple-deficient strain. Upon deletion of *uvrB* from *uvrA⁻mutS⁻* strain, the mutation frequency declines, but remains higher than in *mutS⁻* or in *uvrB⁻mutS⁻* (Table 1; Reference I, Table 2). These results suggest that the 11-fold increase in occurrence of Phe^+ revertants is dependent on UvrB and therefore this phenomenon is likely connected to the role of UvrA in NER and/or functionality of UvrB and UvrC in the absence of UvrA.

Table 1. Effects of NER and UvrA2 on the frequency of the appearance of Phe^+ revertants in growing populations

Strain	Mutation frequency[a] per 1×10^9 cells (median)	Phenotypic effect Ratio/*mutS⁻*
WT	0.8	ND[b]
mutS⁻	50	1
uvrA⁻mutS⁻	562	11[c]
uvrA2⁻mutS⁻	63	1.3
uvrA2⁻uvrA⁻mutS⁻	325	6.5[c]
uvrB⁻mutS⁻	50	1
uvrC⁻mutS⁻	42	0.8
uvrA⁻uvrB⁻mutS⁻	80	1.6[c]

[a] The mutation frequency per 1×10^9 cells in growing cultures was calculated using the Lea–Coulson method of the median (Rosche & Foster, 2000).
[b] ND, not determined.
[c] Denotes statistically significant difference from *mutS⁻* ($P < 0.05$) at the 95% confidence level based on Mann-Whitney test (Sokal, 1981).

NER can affect the mutation frequency in two distinct manners: (1) by leaving mutagenic damage unrepaired or (2) by introducing mutations during repair synthesis step. When mutations are introduced during the repair, the amount of repair events should be in positive correlation with the amount of mutations observed. As deletion of UvrB or UvrC from *mutS⁻* background does not increase the mutation frequency, the effect seen in *uvrA⁻mutS⁻* is not due to leaving mutagenic adducts unrepaired, but due to gratuitous repair. In the absence of UvrA, UvrB and UvrC carry out increased amount of repair reactions, incising either undamaged DNA or damage that in the presence of UvrA would not be recognized as a damage.

P. putida encodes an UvrA-like protein, UvrA2, which is not functional in NER, but can transiently interact with UvrB and shares some functions with UvrA (See the section above: "The role of UvrA2"). In order to find out whether UvrA2 influences the occurrence of NER in *P. putida* cells we

determined the mutation frequency in MutS-deficient strains lacking either UvrA2 or both of the UvrA homologues. The absence of UvrA2 alone does not have a significant effect on the mutations in MMR-deficient bacteria, but loss of *uvrA2* from *uvrA⁻mutS⁻* results in about twofold reduction in mutation frequency (Table 1; Reference I, Table 2). This suggests that indeed, UvrA2 influences the NER process. The twofold reduced mutation frequency in *uvrA2⁻ uvrA⁻mutS⁻* compared to *uvrA⁻mutS⁻* indicates that deletion of *uvrA2* reduces the amount of repair reactions. Thus, UvrA2 acts to even further increase the occurrence of gratuitous repair by UvrB and UvrC, the observation which is conferred by 6.5-fold higher mutation frequency in *uvrA2⁻uvrA⁻mutS⁻* triple mutant compared to *uvrB⁻mutS*.

Based on these results we hypothesize that in the absence of UvrA in *P. putida* UvrB and UvrC carry out gratuitous repair. UvrA and UvrA2 have opposite effects in NER pathway. UvrA leads to more accurate repair by recruiting UvrB and UvrC at 'real' damages and thus reduces gratuitous repair events. UvrA2, on the contrary, shifts the balance towards gratuitous repair.

1.3.2. Role of nucleotide excision repair in mutagenesis in stationary-phase population of *P. putida*

Our investigations on the role of NER in stationary mutagenesis addressed the accumulation of base substitutions and frameshift mutations separately in WT, NER-deficient and UvrA2-deficient strains and in their MMR-deficient derivatives. Building further upon the observations made in the previous section that in *P. putida* UvrB and UvrC may carry out the NER reaction without involvement of UvrA, I will describe in this section: (1) the role of NER in general in mutagenesis (UvrB- and UvrC-deficient bacteria) and (2) the role of UvrA and UvrA2 in this process.

The loss of NER function generally reduces the occurrence of mutations in stationary-phase. The accumulation frequency of base substitutions exhibits a threefold and that of frameshift mutations a sixfold decline in an UvrB-deficient strain compared to the WT (Figures 18A and 18B, Table 2; Reference I, Figures 2A and 2B, Table 3). The absence of UvrC reduces the accumulation rate of both types of mutations by about twofold (Figures 18A and 18B, Table 2; Reference I, Figures 2A and 2B, Table 3).

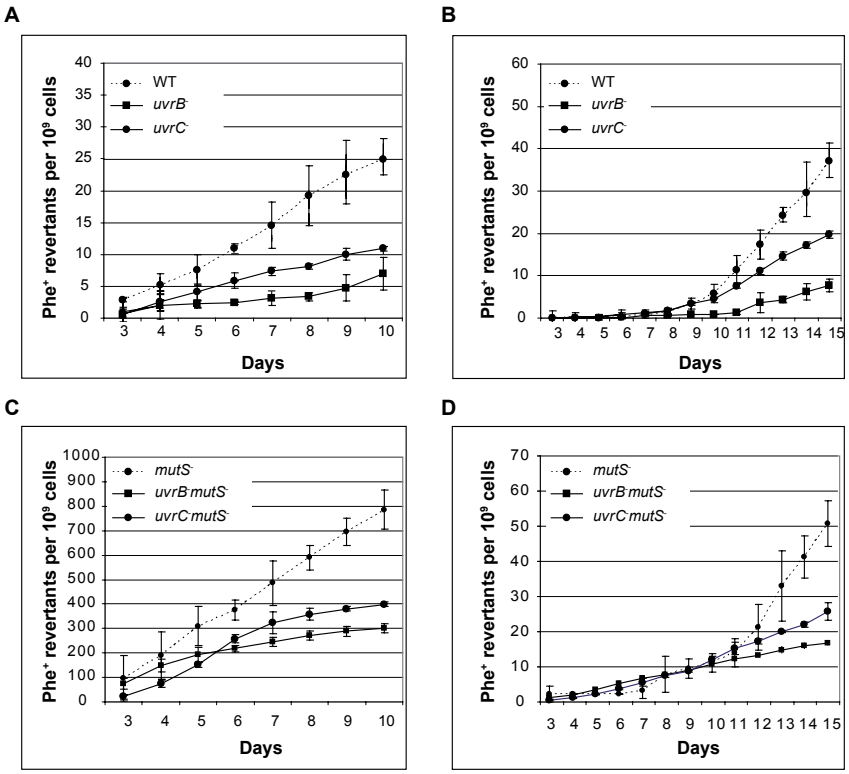


Figure 18. Accumulation of Phe$^+$ revertants on phenol minimal plates in WT and *mutS*$^-$ strains and in their *uvrB*- and *uvrC*-deficient derivatives. **A.** Effect of NER on accumulation of base substitution mutants **B.** Effect of NER on accumulation of 1-bp deletion mutants **C.** Effect of NER on accumulation of base substitution mutants in *mutS*$^-$ background **D.** Effect of NER on accumulation of 1-bp deletion mutants in *mutS*$^-$ background. About 5×10^8 *P. putida* cells either carrying the plasmid pKTpheA22TAG or pKTpheA56 + A were plated from overnight cultures in liquid M9 medium onto phenol minimal plates. Data for at least three parallel experiments are presented. In all cases, the mean ± standard deviation (error bars) for at least 30 plates calculated per 1×10^9 cells is shown.

In MMR-deficient bacteria (*mutS*$^-$) the absence of UvrB or UvrC also negatively affects both types of mutations (Figures 18C and 18D, Table 2; Reference I, Figures 3B and 3D, Table 3). In the case of deficiency of UvrB, again, the accumulation of 1-bp deletion mutants is more severely impaired compared to the accumulation of base substitutions. Whereas the lack of UvrB results in a 2.6-fold decline in the accumulation of base substitution mutants, the accumulation of 1-bp deletion mutants is up to seven times impaired in *uvrB*$^-$*mutS*$^-$ in comparison with *mutS*$^-$. Similar results were obtained for *uvrC*$^-$ both in MutS-deficient and in MutS-proficient backgrounds: inactivation of *uvrC* gene causes a twofold decline in base substitutions and less than a threefold decline in frameshift mutation

frequency compared to WT. We cannot explain the difference in mutation frequencies observed between UvrB- and UvrC-deficient strains. As UvrB in addition to its function in NER plays a role in recombination (Hanada *et al*, 2000), loss of UvrB may also influence the occurrence of recombination and influence the occurrence of mutations in this process.

Table 2. Effects of NER on the frequency of the appearance of Phe^+ revertants in stationary-phase populations

Strain	Base substitutions[a]		1-bp deletions[b]	
	Mutants per day per $1x10^9$ cells	Ratio/WT	Mutants per day per $1x10^9$ cells	Ratio/WT
WT	3.1	1	6.3	1
uvrB$^-$	0.9	0.3[c]	1.0	0.16[c]
uvrC$^-$	1.4	0.45[c]	3.5	0.6[c]
		Ratio/*mutS*		**Ratio/*mutS***
mutS$^-$	98	1	8.0	1
uvrB$^-$*mutS*$^-$	38	0.4[c]	1.2	0.15[c]
uvrC$^-$*mutS*$^-$	50	0.5[c]	2.8	0.35[c]

[a] Average accumulation rate of revertants per day per 1×10^9 cells on days 3–10. There is no significant additional increase in accumulation of base substitution mutants if a later period (days 11–15) is analyzed.

[b] Average accumulation rate of 1-bp deletion mutants per day per 1×10^9 cells is shown only for late starvation period (days 11–15) when the mutation frequency was remarkably increased compared to the earlier starvation period.

[c] Denotes statistically significant difference from WT or *mutS*$^-$ ($P < 0.05$).

Our results clearly show that NER is important in generation, but not in avoidance of stationary-phase mutations. Stationary-phase mutations occur under conditions when growth of bacteria is restricted and chromosome replication is rare. Thus, DNA synthesis which occurs during the course of DNA repair may be an important source for generation of stationary-phase mutations.

The need for NER in repair of oxidative or alkyl damage might be more important in stationary-phase populations. Although bacteria harbor specific glycosylases for removal of each specific type of alkyl or oxidative damage, it might be costly for a cell to synthesize the whole repertoire of repair proteins under nutrient limitation. Given that NER consists of only three repair-specific proteins that are sufficient to remove a large fraction of the occurring lesions, this seems even more likely. In addition to the potential increase in repair in resting cells, NER may also attack undamaged DNA. Branum *et al*. have shown that both *E. coli* and human DNA repair excision nucleases attack undamaged DNA *in vitro* (Branum *et al*, 2001). It has been suggested that such gratuitous excision and the inevitable repair synthesis that follows is a potential source of

spontaneous mutations. In stationary-phase cells when chromosome replication is diminished it could be an essential source of mutations needed for adaptation.

Role of UvrA and UvrA2

Accumulation of base substitution and frameshift mutations in an UvrA-deficient strain is close to that measured in the WT (Figure 19A and 19B, Table 3; Reference I, Figure 2 and Table 3). This data further supports the observation made in growing cultures, that UvrA is not needed for mutagenic NER. We also studied the effects of the absence of UvrA in MMR-defective strain. The results presented in Figures 19C and 19D, Table 3; Reference I, Figures 3A and 3C show that the frequency of accumulation of stationary-phase mutations increases by approximately tenfold in the case of both assay systems if UvrA is absent in a *mutS*$^-$ background. Moreover the knockout of *uvrB* in a *uvrA*$^-$*mutS*$^-$ strain reduces the mutation frequency close to that of the *mutS*$^-$ strain (Figures 19C and 19D, Table 3; Reference I, Figures 3A and 3C, Table 3). These data indicate that the UvrA-dependent mutation avoidance pathway functions in the similar manner in starving and in growing bacteria.

The lack of UvrA2 in starving bacteria reduces the accumulation of base substitution mutations, but the frequency of 1-bp deletions does not change compared to the WT (Figures 19A and 19B, Table 3; Reference I, Figure 2 and Table 3). The effect of UvrA2 on base substitution mutation is completely abolished in the *uvrA2uvrA* double-deficient strain, showing that occurrence of base substitution mutations facilitated by UvrA2 is counteracted by UvrA (Figures 19A and 19B, Table 3; Reference I, Figure 2 and Table 3).

In contrast to UvrA, the absence of UvrA2 in a MutS-deficient background drastically reduces the accumulation of 1-bp deletion mutants in starving populations of bacteria. This demonstrates that the presence of UvrA2 facilitates the occurrence of stationary-phase mutations. The accumulation rate of frameshift mutations is 27-fold lower in *uvrA2*$^-$*mutS*$^-$ compared to *mutS*$^-$ (Figure 19D and Table 3; Reference I, Figure 3D and Table 3). 1-bp deletions are reduced in MutS-deficient bacteria also in the case that both UvrA homologues are absent: the Phe$^+$ revertants accumulate in *uvrAuvrA2mutS* triple-deficient strain at a 3.6-fold lower frequency than in *mutS*$^-$. This mutation frequency is 27-fold lower than that in the *uvrAmutS* double-deficient strain (Table 3; Reference I, Table 3). These data suggest that UvrA2 has a large impact on the occurrence of 1-bp deletions in the absence, but also in the presence of UvrA.

The fact that UvrA2 negatively affects occurrence of base substitutions as well, becomes evident only in *uvrAmutS* double-deficient strain. Deletion of *uvrA2* from *uvrA*$^-$*mutS*$^-$reduces occurrence of base substitution mutations 3.5-fold (Table 3; Reference I, Figures 3A and 3B, Table 3). No effect of UvrA2 is seen in the occurrence of base substitution mutations in UvrA-proficient strain in *mutS*$^-$ background.

Taken together, our results imply that UvrA and UvrA2 have opposite roles not only in growing cells, but also in stationary-phase bacteria. While UvrA acts to reduce mutagenesis, UvrA2 facilitates the occurrence of mutations.

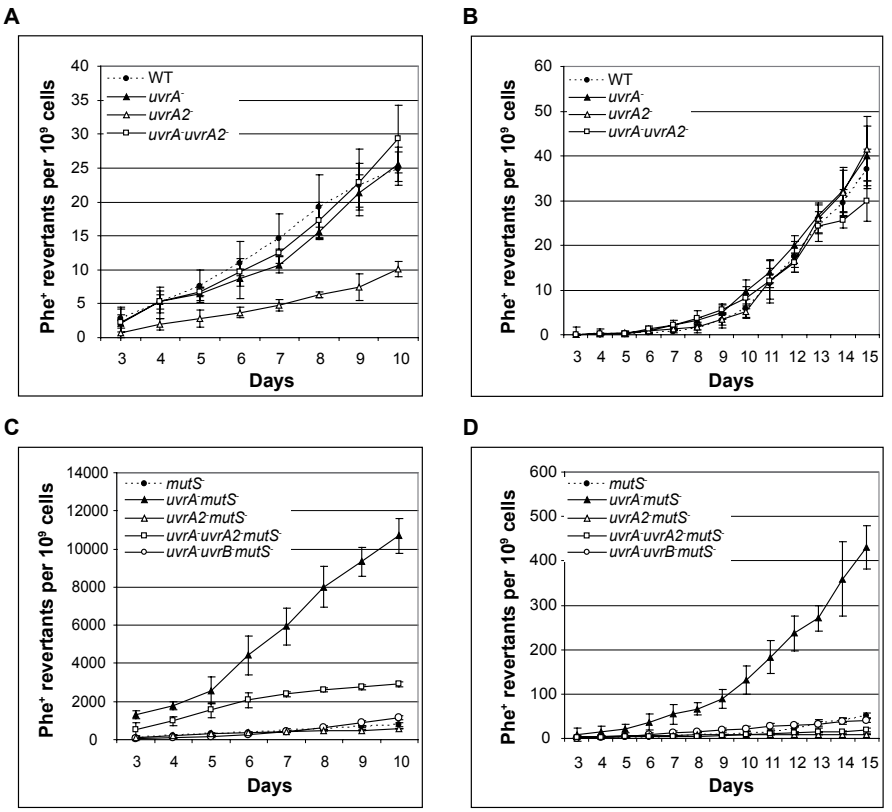


Figure 19. Accumulation of Phe$^+$ revertants on phenol minimal plates in WT and *mutS*$^-$ strains and in their *uvrA*- and *uvrA2*-deficient derivatives. **A.** Effect of UvrA and UvrA2 on the accumulation of base substitution mutants **B.** Effect of UvrA and UvrA2 on the accumulation of 1-bp deletion mutants **C.** Effect of UvrA and UvrA2 on the accumulation of base substitution mutants in a *mutS*$^-$ background **D.** Effect of UvrA and UvrA2 on the accumulation of 1-bp deletion mutants in a *mutS*$^-$ background. About 5×10^8 *P. putida* cells either carrying the plasmid pKTpheA22TAG or pKTpheA56 + A were plated from overnight cultures in liquid M9 medium onto phenol minimal plates. Data for at least three parallel experiments are presented. In all cases, the mean ± standard deviation (error bars) for at least 30 plates calculated per 1×10^9 is shown.

Table 3. Effects of UvrA and UvrA2 on the frequency of the appearance of Phe$^+$ revertants in stationary-phase populations

Strain	Base substitutions[a]		1-bp deletions[b]	
	Mutants per day per 1x10^9 cells	Ratio/WT	Mutants per day per 1x10^9 cells	Ratio/WT
WT	3.1	1	6.3	1
uvrA$^-$	3.2	1	6.0	1
uvrA2$^-$	1.2	0.4[c]	7.3	1.2
uvrA2$^-$ *uvrA*$^-$	3.7	1.2	4.3	0.7
		Ratio/*mutS*		**Ratio/*mutS***
mutS$^-$	98	1	8.0	1
uvrA$^-$*mutS*$^-$	1249	12.7[c]	60	7.5[c]
uvrA2$^-$*mutS*$^-$	69	0.7	0.3	0.04[c]
uvrA2$^-$*uvrA*$^-$ *mutS*$^-$	364	3.7[c]	2.2	0.28[c]
uvrA$^-$*uvrB*$^-$*mutS*$^-$	145	1.5[c]	1.4	0.18[c]

[a] Average accumulation rate of revertants per day per 1×10^9 cells on days 3–10. There is no significant additional increase in accumulation of base substitution mutants if a later period (days 11–15) is analyzed.

[b] Average accumulation rate of 1-bp deletion mutants per day per 1×10^9 cells is shown only for late starvation period (days 11–15) when the mutation frequency is remarkably increased compared to the earlier starvation period.

[c] Denotes statistically significant difference from WT or *mutS*$^-$ ($P < 0.05$).

Although NER is considered an error-free process when repairing UV-induced damage, we have found that this repair pathway facilitates the occurrence of mutations in resting cells. These mutations may be essential in adapting to growth in changing environments. We propose that the mutations are introduced during the repair synthesis carried out by DNA pol I or some specialized DNA polymerase.

The idea that repair by NER can be erroneous is not new. After the finding that NER can excise oligomers of 12–13 nucleotides from undamaged DNA with a low but significant efficiency *in vitro* (Branum *et al*, 2001), more attention has been drawn to mutagenesis potentially induced by the repair. The fact that NER can excise undamaged DNA led to two linked hypotheses for the mechanism underlying mutagenic repair: (1) the damage discrimination properties of NER are not stringent enough to totally impede attacking undamaged DNA; (2) mutations occur during repair synthesis carried out by DNA pol I or some other DNA polymerase. *In vivo* evidence that NER indeed causes an increase in mutation frequency has emerged only recently. About the same time we demonstrated mutagenic NER in *P. putida* stationary-phase populations, it was proven that NER enhances the occurrence of mutations induced by oxidized nucleotides in *E. coli* (Hori *et al*, 2007). As the amount of oxidative damage is considered to increase in resting bacteria our findings might also be connected to the action of NER on oxidatively damaged nucleotides in the DNA.

Recently Hasegawa *et al* showed, using a set of *E. coli* NER and DNA pol I-deficient strains, that the increase in NER-dependent mutagenesis is a direct consequence of the repair reaction and synthesis carried out by DNA pol I (Hasegawa *et al*, 2008). However, DNA pol I may not be the only polymerase participating in repair synthesis. Specialized polymerases have also been shown to carry out DNA synthesis on short gaps and due to their low fidelity mutations can be easily introduced. Moreover, co-operation of DNA polymerases IV and V and immediate fixation of mutations has been suggested on gaps generated during NER if both of the DNA strands contain closely situated lesions (Fuchs, 2009).

Different from the situation observed in *E. coli*, UvrA does not participate in mutagenic NER in *P. putida*. Our results indicate that mutagenic NER is carried out solely by UvrB and UvrC. This process is enhanced in the presence of UvrA2, the UvrA homologue that by itself is not able to participate in NER reaction. In which manner UvrA2 affects the NER process is not known. As the protein can transiently interact with UvrB (Timmins *et al*, 2009), it can possibly recruit UvrB to distortions in DNA otherwise not recognized as a damage. UvrA, on the contrary, acts as a specificity factor in NER, preventing unnecessary repair reactions from occurring.

II MUTAGENIC POTENTIAL OF DAMAGE TOLERANCE MECHANISMS UNDER STARVATION STRESS

DNA damage tolerance is a way of dealing with damage without its removal. It allows immediate continuation of DNA synthesis and progression of the cell-cycle while the lesion can be removed by repair mechanisms only later. DNA damage tolerance is achieved by single-strand DNA gap filling and translesion synthesis (TLS) carried out by specialized DNA polymerases (both of these processes are described in the literature review chapter). While the gap filling process is generally error-free, TLS is carried out by specialized DNA polymerases and may cause mutations on the replicated DNA. In the Results and Discussion chapter of this thesis I will only consider the role of specialized DNA polymerases in damage tolerance and the effects these polymerases have on mutational processes. Specialized DNA polymerases, although often called error-prone are not erroneous *a priori* (Jarosz *et al*, 2007). They replicate across certain types of damage in an error-free manner, but insert a 'wrong' nucleotide opposite other types of lesions, with each of the specialized DNA polymerases (for instance in *E. coli* DNA pol II, pol IV and pol V) having different specificity. Due to their potentially error-prone nature, expression of the specialized DNA polymerases is under tight control of the SOS system. This regulation assures that the polymerases are expressed and have access to the replication only if needed. The SOS response is induced when bacteria encounter severe

DNA damage. It is also constitutively induced in other non-favourable conditions such as starvation during the stationary-phase. Under such conditions specialized DNA polymerases contribute to mutagenesis in *E. coli* and are essential for providing growth fitness in aged populations (Bhamre *et al*, 2001; Yeiser *et al*, 2002).

DNA pol V encoded by *umuDC* is the only DNA polymerase in *E. coli* capable of carrying out TLS across UV-induced DNA damage. In that process, DNA pol V is highly erroneous. Differently from enterobacteria, *umuDC* orthologs encoding DNA pol V are absent from genomes of most bacterial species studied, even those of phyllospheric habitat. Species from the *Pseudomonas* genus are also among those lacking a chromosomally encoded DNA pol V homologue. Instead, a multiple gene cassette *lexA2-imuA-imuB-dnaE2* encoding two putative DNA polymerases, ImuB and DnaE2, exists in the genome of *Pseudomonas putida*. This operon is damage-inducible and under negative control of the LexA homologue (LexA2) encoded by the same gene cassette (Abella *et al*, 2004). This so called "mutagenesis cassette" is present not only in *Pseudomonas* species but is widely spread in the genomes of bacteria lacking DNA pol V (Erill *et al*, 2006a). The non-simultaneous presence of *umuDC* orthologs encoding DNA pol V and the "mutagenesis cassette" in bacterial genomes has led to suggestion that this gene cassette replaces the functions of *umuDC* in bacteria lacking DNA pol V (Galhardo *et al*, 2005; Erill *et al*, 2006a). However, evidence in support of that suggestion is still inadequate due to the scarce amount of experimental data.

Although DNA polymerase V is not encoded in the chromosomes of the majority of sequenced bacterial species, genes homologous to *umuDC* are spread in broad host range plasmids. These plasmids carry genes for various degradative pathways and provide bacteria with the ability to utilize different substrates as a carbon source. While degradation genes in these plasmids are nested in transposons and can be easily deleted from the plasmid or rearranged, the genes sharing homology with *umuDC* are clustered together with the maintenance functions of the plasmid in the core area. Of the plasmids isolated from natural isolates of *Pseudomona*s about 1/3 confer UV-tolerance and UV-induced mutability to their host (Jacoby *et al*, 1983). These properties have been shown by others and by us to be connected to the presence of *umuDC* homologues on the plasmid (Herrera *et al*, 1988; Sundin *et al*, 1996; Reference IV).

The natural simultaneous presence of DNA pol V genes on plasmid and the "mutagenesis cassette" in the chromosome of *Pseudomonas putida* makes it a unique model system for studying similarities and differences of the two pathways under conditions of UV exposure as well as at mutagenesis in resting cells. This study thus could shed light on the question why DNA pol V is encoded in plasmids instead of the chromosome and why the presence of a "mutagenesis cassette" is favored by many bacterial species.

2.1. "mutagenesis cassette"

2.1.1. Role of the "mutagenesis cassette" upon exposure of *P. putida* to UV radiation (Reference II)

The aim of this study was to investigate whether DNA polymerases encoded in the mutagenesis cassette could substitute the functions of DNA pol V in *P. putida*. DNA polymerase V is known to increase the tolerance of bacteria to UV radiation and carry out highly erroneous TLS across UV radiation-induced DNA damage. We have studied the role of "mutagenesis cassette" on the UV-tolerance and UV mutagenesis phenotypes of *P. putida* by comparing the expression of these phenotypes in *P. putida* wild type (WT) strain (plasmid free), in its *dnaE2*- or *imuB*-deficient derivatives and in a *dnaE2imuB* double-deficient strain.

Role of DnaE2 and ImuB in UV-tolerance

For assessing the role of DnaE2 and ImuB in UV-tolerance we exposed the cells of *P. putida* WT and its *dnaE2*- and/or *imuB*-deficient derivatives to different doses of UV-C radiation and calculated the fraction of surviving colony forming units (CFU). Deficiency in *dnaE2* and *imuB* as well as simultaneous deficiency in both genes results in similar viability compared to WT at all UV doses used. All the studied strains tolerate the UV-C dose of 10 J/m^2 with no detectable reduction in viability, whereas survival is reduced to almost zero after the exposure to a dose of 50 J/m^2 (S. Saumaa, personal communication).

We anticipated a slight decline in viability in DnaE2-deficient strain upon UV exposure. Although UV-tolerance of *dnaE2* has not been studied in other organisms, the polymerase has been shown to replicate across UV induced lesions (Boshoff *et al*, 2003). The absence of TLS polymerase DNA pol V causes decline in viability of bacteria upon UV radiation (Friedberg *et al*, 2006). However, it is still unknown whether the increased susceptibility of an *E. coli umuDC*-deficient strain to UV radiation is caused by loss of the DNA polymerase capable of continuing replication over encountered damage or by loss of the cell-cycle delay provided by $UmuD_2C$ (Murli *et al*, 2000). The delay in cell cycle facilitates the direct removal of the DNA damage before the cell attempts to continue its genome replication. On the other hand, even if replication by DNA pol V is indeed crucial for providing tolerance to UV radiation in *E. coli*, the lack of the TLS polymerase activity may not cause similar effects in other species. Bacteria have different partially redundant systems for dealing with UV-induced damage. The sensitivity caused by the absence of one system depends on the efficiency of other DNA repair or tolerance systems capable of dealing with the same type of damage. *P. putida* strains deficient in *dnaE2* and/or *imuB* have photolyase activity and are proficient in NER. These repair pathways can be sufficient to remove all the

damage in the case when TLS polymerase is not available and therefore no loss in tolerance to UV radiation is encountered.

UV sensitivity caused by the absence of DnaE2 and ImuB became apparent only when the experiment was repeated in a DNA pol I (*polA*) deficient background (S. Saumaa, personal communication). DNA pol I is known to participate in Okazaki fragment synthesis and in the gap filling reaction in NER. Lack of either DnaE2 or ImuB results in the *polA*-deficient strain being killed even upon exposure to very low UV-C doses, such as 10 J/m^2. Equal effect of DnaE2 and ImuB on viability of *polA*-deficient strain suggests that DnaE2 and ImuB function together in the replication process. The notion that these proteins function together is in line with observations in *Caulobacter crescentus*. In this species deletion of genes for ImuB, ImuA or DnaE2 led to a similar loss of UV mutagenic phenotype; effect on UV-tolerance was not reported in this study (Galhardo *et al*, 2005).

It is notable that *polAdnaE*- and *polAimuB*-deficient strains exhibit survival pattern similar to that of NER-deficient strains (*uvrB*$^-$ and *uvrC*$^-$) in response to UV radiation (Reference I). This leads us to hypothesize that DnaE2 participates in repair synthesis in NER analogous to DNA pol I and that simultaneous inactivation of these polymerases causes deficiency in NER.

DnaE2 and ImuB have opposite effects on UV-induced mutagenesis

In order to further investigate the possible participation of DnaE2 and ImuB in replication across UV-induced lesions, we studied the UV-mutagenic phenotype of the strains deficient either in *dnaE2* or *imuB* or in both of the putative DNA polymerases. Rif^R assay was used to monitor occurrence of mutations. All the studied strains exhibit similar spontaneous mutation frequency (Table 4). In response to exposure to UV radiation, the mutation frequency in WT increases twofold. The mutation frequencies observed in *dnaE2*- and *imuB*-deficient strains after UV-exposure were unexpected. The lack of DnaE2 does not reduce the mutation frequency but instead results in a threefold increase compared to the WT (Table 4, Reference II, Figure 3). This indicates that DNA synthesis by DnaE2 in *P. putida* does not cause UV-induced mutagenesis but averts it. However, using the same Rif^R assay it was shown in *Mycobacterium* species that DnaE2 is the polymerase responsible for tenfold increase in mutation frequency upon UV radiation (Boshoff *et al*, 2003). The second DNA polymerase III subunit PolC is responsible for UV-induced mutagenesis also in *P. aeruginosa* (Sanders *et al*, 2006).

Differently from the DnaE2-deficient strain, the ImuB-deficient mutant exhibits no increase in the mutation frequency in response to UV radiation compared to the level of spontaneous mutations. Moreover, ImuB-deficient strain exhibits approximately twofold lower mutation frequency than WT and about 5.5-fold lower mutation frequency than the strain lacking DnaE2. At the

same time, the *imuB* and *dnaE2* double-deficient strain shows a mutation frequency comparable to that of *imuB* after exposure to UV (Table 4; Reference II Figure 3). These findings suggest that: (1) ImuB is required for DNA synthesis carried out by DnaE2, as additional deletion of DnaE2 from an ImuB-deficient strain does not lead to any changes in UV-induced mutation frequency and (2) in addition to DnaE2 ImuB may facilitate some other unidentified DNA polymerase to carry out error-prone replication. We propose that ImuB functions as a scaffold protein mediating polymerase traffic at the site of replication. Such a role is further supported by the fact that ImuB lacks the amino acids essential for forming the catalytic centre (Reference II, Supplementary figure 1). In fact, the idea that certain DNA polymerases can facilitate access of other polymerases to replication is established for several DNA polymerases. A good example is eukaryotic Y family polymerase REV1. The catalytic activity of this enzyme is relatively poor (Choi & Guengerich, 2008). As REV1 interacts with PCNA and DNA polymerases η, κ, ι, and ζ, it has been suggested to serve as a scaffold protein for the recruitment of polymerases (Guo *et al*, 2003; Guo *et al*, 2006). The other example is, for instance, the interplay between DNA polymerases III and IV described in the literature review chapter of this thesis.

Table 4. Contribution of DnaE2 and ImuB to UV-induced mutagenesis

Strain	frequency of spontaneous mutations	frequency of UV-induced[a] mutations
	(Rif^R mutants per 10^9 cells)	
WT	**1.9**	**3.7**
dnaE2	**1.7**	**10.5[b]**
imuB	**2.2**	**2.2[b]**
imuBdnaE2	**2.1**	**1.9[b]**

[a]Bacteria were exposed to UV-C dose 100 J/m^2 at wavelength 254 nm
[b]Denotes significant difference from WT (*P*-value less than 0.05) at the 95% confidence level based on the Mann-Whitney test (Sokal, 1981).

2.1.2. Role of DnaE2 and ImuB in stationary-phase mutagenesis in *P. putida*

In order to assess the contribution of DnaE2 and ImuB to stationary-phase mutagenesis, we carried out starvation experiments using the Phe$^+$ test-system. The results presented in Figure 20; Reference II, Figure 1 reveal that the absence of functional *dnaE2* gene enhances the appearance of base substitution mutations in starving *P. putida,* but does not affect the appearance of frameshift mutations. Compared to the WT strain, the frequency of accumulation of Phe$^+$ revertants is 2.5–3 times elevated in a DnaE2-deficient strain during the 10-days starvation period studied. Thus, DnaE2 acts as an error-free DNA polymerase which role

can be taken over by other DNA polymerase(s) with lower replication fidelity than that of DnaE2. These results are in accordance with the results of the studies of UV mutagenesis described in the previous section. Notably, fourfold elevated frequency of spontaneous mutations compared to WT strain was also observed in growing cells of the DnaE2-deficient strain using the Phe^+ test-system (data not shown). The fact that the 'antimutator' role of DnaE2 can be seen also in growing cultures not exposed to exogenous DNA damage suggests that this polymerase may have a more general function (possibly shared with DNA pol I). It may participate in genomic DNA replication or in DNA repair synthesis or in both of the processes rather than be a specialized polymerase which replication is restricted mainly to damage conditions.

The lack of ImuB results in up to twofold lower frequency of emergence of both base substitution mutants and 1-bp deletions in starving *P. putida* (Figure 20; Reference II Figure 1). The strain deficient in ImuB and DnaE2 has mutation frequency similar to that of *imuB*$^-$ when base substitution mutations are assessed. In frameshift mutations twofold decline is observed (Figure 20; Reference II Figures 1 and 2). The loss of DnaE2 effect in *dnaE2imuB* double-deficient strain further confirms results from the UV mutagenesis studies that DnaE2 is a polymerase which DNA synthesis capability is dependent on ImuB. The data obtained with test-system counting for frameshift mutations indicates that DnaE2 is not the only DNA polymerase that is recruited to replication by ImuB. The yet unidentified polymerase engaged in this process is likely error-prone and its effect on the occurrence of frameshifts is likely larger than that of base substitution mutations.

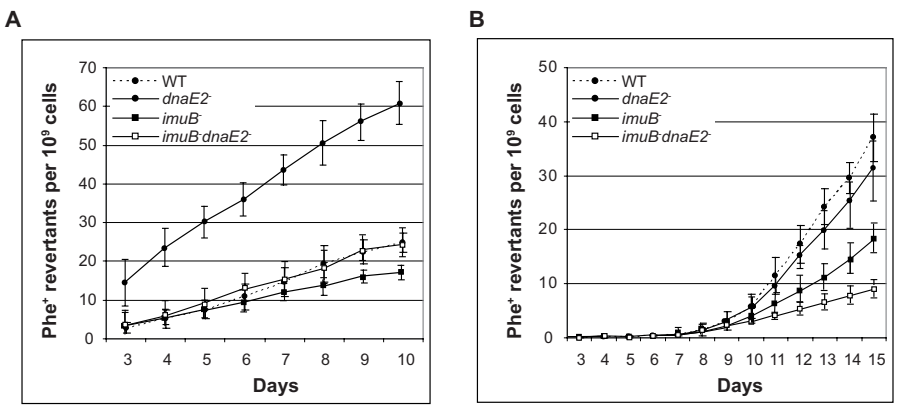


Figure 20. Accumulation of Phe^+ revertants on phenol minimal plates in *P. putida* WT strain and in its *dnaE2*- or *imuB*-deficient derivatives. **A.** Effect of DnaE2 and ImuB on accumulation of base substitution **B.** Effect of DnaE2 and ImuB on accumulation of 1-bp deletion mutants. About 5×10^8 *P. putida* cells were plated onto phenol minimal plates from overnight liquid M9 medium-grown independent cultures. Data for at least five parallel experiments are presented. In all cases, means ± S.D. (error bars) for at least 10 plates calculated per 1×10^9 cells are shown.

In conclusion, our results suggest that DnaE2 in *P. putida* is a high fidelity DNA polymerase which recruitment to DNA replication is dependent on ImuB. The polymerase does not confer an UV-induced mutagenesis phenotype nor increase the tolerance of bacteria to UV radiation. Therefore DnaE2 does not reveal any capability of substituting DNA pol V in this species. Instead, the polymerase may assist DNA pol I in lagging strand synthesis and/or in DNA repair synthesis. Additionally, DnaE2 may be a polymerase specialized for translesion synthesis over lesions other than those caused by UV radiation or oxidative damage in starving bacteria.

2.2. DNA polymerase V homologues encoded on large degradative plasmids (Reference IV)

Bacteria from the genus *Pseudomonas* do not encode DNA polymerase V in their genome. This can be one of the reasons why in general pseudomonads are relatively sensitive to UV and also do not express an UV-mutable phenotype. Many large plasmids carrying genes of different catabolic pathways increase the survival and mutation rate of their host upon exposure to UV irradiation (Jacoby *et al*, 1983). These phenotypes are in *Pseudomonas* species connected to *rulAB* genes homologous to DNA pol V-encoding *umuDC* in *E. coli* (Sundin *et al*, 1996). The plasmids encoding the *rulAB* operon are essential for fitness of leaf colonizing *Pseudomonas* species *P. syringae* and *P. fluorescence* in sun-exposed environments. It is plausible that the benefit in UV-tolerance provided by expressing the *rulAB* genes makes *P. syringae* one of the most ubiquitous bacterium in phyllosphere (Lindow & Brandl, 2003). In fact, all Pseudomonas strains isolated so far from sun-exposed habitats carry large plasmids that encode in their core a functional *rulAB* operon (Sundin & Bender, 1996). The hypothesis is strengthened by the fact that upon transfer of the *rulAB* operon to *P. aeruginosa*, a bacterium otherwise relatively sensitive to UV and not fit on leaf surfaces, exhibits enhanced fitness and can persist extended time on leaf surface (Cho *et al*, 1975).

It is interesting to note that *Pseudomonas* species isolated from the soil habitats not exposed to sun also carry plasmids conferring UV-resistant phenotype (Jacoby *et al*, 1983). As these large plasmids harbor genes for degradation of aromatic compounds, *rulAB*-encoded proteins may be of benefit not only under conditions of continuous sun exposure, but also increase tolerance of bacteria towards potentially toxic compounds and intermediates accumulating during degradation. For instance, aromatic agents such as benzene induce DNA damage in eukaryotic cells (Moller, 2005). It has also been shown that the pPL1 plasmid which increases the UV-tolerance of *P. aeruginosa* also protects the cells from X-ray and freeze-thaw damage (Williams & Calcott, 1982).

The TOL plasmid pWW0 isolated from *P. putida* strain mt-2 (Williams & Murray, 1974) is one of the most thoroughly studied catabolic plasmids. It consists of a catabolic region where the toluene degradation genes are situated

in two nested transposons, and of a region that carries plasmid functions, i.e. functions needed for replication, maintenance and transfer. This 46 kb region encoding plasmid functions is named IncP-9 core of the plasmid (Greated *et al*, 2002). Analysis of the pWW0 sequence has revealed that the plasmid core carries *ruvAB* genes homologous to *umuDC* genes encoding DNA pol V in *E. coli*. *ruvA* (Q8VMP5) and *ruvB* (Q8VMP6) are closely related to *rulA* (Q52416) and *rulB* (Q52417), respectively, identified in *P. syringae* plasmid pPSR1 (Sundin *et al*, 2004b). The two-gene operon confers UV-tolerance on *P. syringae* and complements *E. coli umuDC* mutant in UV mutagenesis (Kim & Sundin, 2000; Sundin & Murillo, 1999). The designation of the genes homologous to *umuDC* as *ruvAB* in pWW0 is misleading as the genes designated as *ruvA* and *ruvB* are also found in the chromosomes of bacteria. The chromosomal *ruvA* and *ruvB* encode for a Holliday junction helicase complex necessary for branch migration along the DNA. To avoid confusion between chromosomally and plasmidially encoded *ruvAB* genes we decided to rename the plasmidial *ruvAB* to *rulAB* as the genes exhibit highest similarity to *P. syringae* pPSR1 *rulAB* genes (Reference IV). We have characterized the role of the DNA pol V homologue encoded on the toluene degradation plasmid pWW0 in tolerance to UV-induced damage and in mutational processes of *P. putida*.

2.2.1. *rulAB* genes from the TOL plasmid pWW0 confer UV-tolerance and UV-mutability

Transferring the TOL plasmid pWW0 into the plasmid-free *P. putida* WT strain results in an increased tolerance of bacteria to UV radiation. While only 10^{-4} fraction of bacteria survives after exposure to UV-C at 50 J/m^2, the presence of the TOL plasmid increases the survival fraction by tenfold, to 10^{-3} (Table 5; Reference IV, Figure 2). In addition, bacteria harboring the TOL plasmid exhibit sevenfold increased UV-mutability compared to the plasmid-free WT strain. A comparable (approximately tenfold) TOL plasmid-dependent increase in mutation frequency is also observed when bacteria are treated with a DNA crosslinking chemical mitomycin C (MMC). Notably, presence of the TOL plasmid does not affect the frequency of spontaneous mutations (Table 5; Reference IV, Figure 1).

In order to verify that the *rulAB* genes from pWW0 are the genes conferring UV-mutability and increased tolerance to UV radiation, we cloned *rulAB* genes from the TOL plasmid and inserted them into a random location on the *P. putida* chromosome using mini-Tn5tel^R system. Ten clones with different locations of miniTn5 carrying *rulAB* were examined for spontaneous and UV-induced mutability. Nine clones from ten expressed a similar UV-mutagenesis phenotype as WT carrying the TOL plasmid and similar low spontaneous mutation rate. One of these nine clones was selected for further experiments. This strain is further referred to as PaWrulAB.

The constructed PaWrulAB strain tolerated higher doses of UV compared to WT, exhibiting a similar UV-tolerant phenotype as WT carrying the pWW0 plasmid (Table 5; Reference IV, Figure 2). The frequencies of spontaneous and UV-induced Rif^R mutations for *P. putida* either carrying the *rulAB* genes in the chromosome or harbouring the TOL plasmid were similar (Table 5; Reference IV, Figures 1A and 1B). Upon exposure to UV these strains showed more than tenfold increase in mutation frequency compared to the uninduced levels while in WT the increase was only twofold. The rate of mitomycin C-induced mutations was approximately fivefold higher than in the WT strain, but remained twofold lower in PaWrulAB compared to that in WT[pWW0] (Table 5; Reference IV, Figure 1C). In order to confirm that *rulAB* genes are responsible for both, induced mutability and UV-tolerance, we constructed a pWW0 derivative lacking functional *rulAB* genes. As expected, a TOL plasmid with inactivated *rulAB* operon did not confer any increased tolerance towards UV radiation nor an increased UV mutagenesis phenotype (Reference IV, data not shown).

Our results clearly indicate that the UV-induced mutability and UV-tolerance phenotype conferred by TOL plasmid pWW0 is determined by *rulAB* genes. The expression of these genes does not seem to be significantly influenced by other genes on pWW0.

Table 5. UV-tolerance and mutagenesis conferred by *rulAB* genes from TOL plasmid

	Survival of bacteria after exposure to UV[a] (% of viable cells)	**Spontaneous mutations**	**UV-induced mutations**	**MMC-induced mutations**
		(Rif^R per 10^9 viable cells)		
WT	0.009	3.6	7.0	769
WT[pWW0]	0.13	3.7	45.4	6875
PaWrulAB	0.1	2.9	38.5	3625
***recA*⁻**	–	–	8.0	–
PaWrulAB*recA*⁻	–	–	8.0	–

[a]Bacteria were exposed to UV-C dose 50 J/m^2 at wavelength 254 nm

Bacterial survival and mutation frequencies were calculated using the Lea-Coulson method of the median (Rosche & Foster, 2000).

– denotes data not determined

2.2.2. Regulation of expression of the pWW0-encoded polymerase V homologue in *P. putida*

As *P. putida* is a bacterium with soil habitat and therefore spends long periods in stationary-phase, we decided to study the expression of the *rulAB* operon through the bacterial life cycle until the late stationary-phase. As chromosomal *P. putida* genes show relatively low induction upon damage (Tegova *et al*, 2004; Abella *et al*, 2007) it was also of interest to analyze the damage-inducibility of the plasmid-born *rulAB* operon.

Transcription initiation from the *rulAB* promoter is damage-inducible

The putative promoter region of the *rulAB* genes is similar to the promoter region of the *umuDC* genes characterized in *E. coli* (Kitagawa *et al*, 1985). A putative SOS box CTGTATATGCAAACAG which perfectly matches the *E. coli* LexA binding consensus $CTGTN_8ACAG$ overlaps the –10 sequence (depicted in Figure 4 of Reference IV). This region may function as a binding site for the LexA repressor at the *rulAB* promoter. In many bacteria studied, DNA damage stimulates a RecA-mediated cleavage of the LexA repressor, resulting in an increased level of transcription of the DNA damage-induced genes (see the section "SOS response in *E. coli*" in the literature review of the thesis). The promoter area of the *rulAB* genes is likely bound by LexA1 as this LexA homologue of *P. putida* binds to the *E. coli*-like LexA binding sequences (Calero *et al*, 1991; Abella *et al*, 2004). In order to study the *rulAB* promoter activity in the presence of DNA damage, we cloned this promoter upstream of the *luxAB* reporter in the broad host range plasmid pRP9TT. The luciferase activities measured in exponentially growing *P. putida* cells containing the promoter construct indicated that the transcription initiation occurs at the promoter of the *rulAB* genes at a basal level even if no DNA damage is induced (Figure 21A; Reference IV, Figure 5A). However, bacteria exposed to MMC for three hours have threefold higher level of luciferase activity than the bacteria grown without the DNA damaging agent. Therefore, transcription of the *rulAB* operon is damage-inducible in *P. putida*. This was the starting-point to further investigate whether transcription from the *rulAB* promoter is regulated by the classical SOS response as described for the *umuDC* operon in *E. coli*. For that purpose, we measured the activity of the *rulAB* promoter with and without MMC in a RecA-deficient derivative of WT strain. In the absence of RecA, the LexA repressor is not inactivated and it does not free the promoter areas of the genes belonging to SOS regulon. We found that in the RecA-deficient *P. putida* strain *rulAB* genes are transcribed in basal level and their expression level does not change upon damage induction (Figure 21B; Reference IV, Figure 5B). These results clearly show the need of RecA to increase *rulAB* expression upon exposure to a DNA damaging agent. The lack of RecA also completely abolishes the effect of *rulAB* on UV mutagenesis although the *rulAB* genes are transcribed at a relatively high basal level (Table 5, Figure 21B; Reference IV, Figures 6 and 5B). In addition to transcriptional level, *E. coli* DNA pol V is controlled by RecA at the posttranslational level by mediating proteolytic cleavage of UmuD to UmuD'. We propose that RulAB is also activated by RecA by proteolytic cleavage of RulA and thus in the absence of RecA no functional polymerase can be formed.

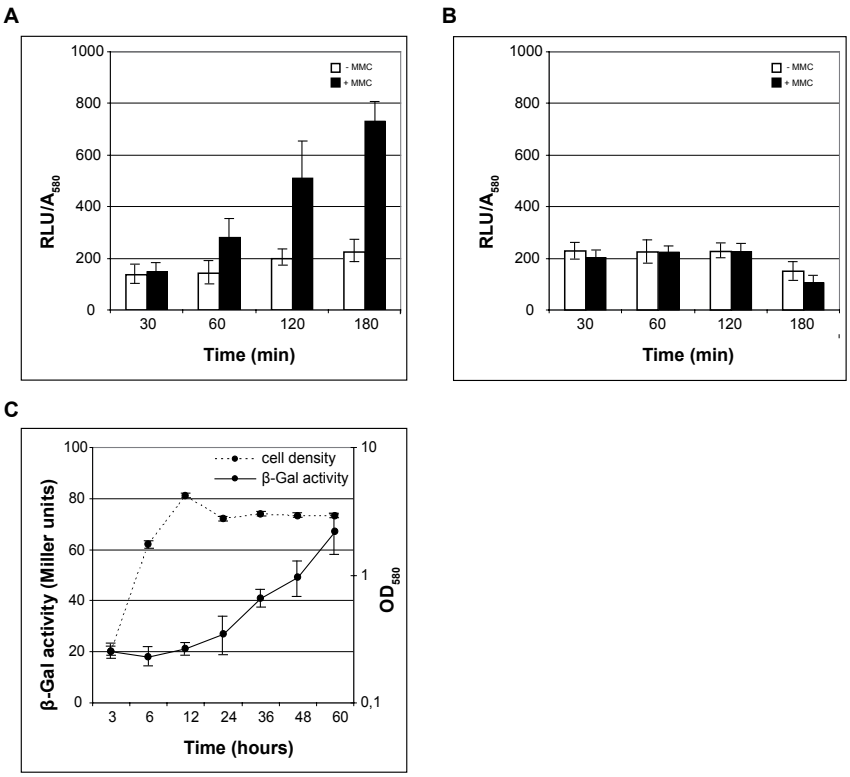


Figure 21. Study of the effect of DNA damage on transcription from the *rulAB* promoter **A.** in *P. putida* WT strain **B.** in its RecA-deficient derivative *recA*⁻. Transcription from the *rulAB* promoter was assayed by measuring the luciferase activity (relative luciferase units/optical density units at 580 nm) in cells harboring pPR9TTPrulABluxAB plasmid containing *rulAB* promoter-*luxAB* fusion and grown in M9 medium supplemented with glucose and CAA in the presence or absence of the DNA-damaging agent MMC (2 μg/ml). **C.** Effect of growth phase of bacteria on transcription from the *rulAB* promoter. *β*-Gal activity was measured in the *P. putida* WT strain carrying the *rulAB* promoter-*lacZ* fusion in plasmid pKTPrulABlacZ. Bacteria were grown in M9 medium supplemented with glucose and CAA. The growth curve of the bacteria is indicated by a dashed line. The results of four independent experiments are presented. The standard deviations are shown on the figure (error bars). OD_{580}, optical density at 580 nm.

RulAB operon is expressed under the conditions of carbon starvation

For studying the effect of the growth phase on the level of transcription from the *rulAB* promoter we used a pRP9TT-based construct with the *lacZ* reporter gene under the control of the *rulAB* promoter. The transcription from the *rulAB*

promoter increases only slightly when cells enter the stationary-phase (Figure 21C, samples at hours 12 and 24; Reference IV, Figure 5C). Surprisingly, the promoter activity continued to increase after these time points, and by late stationary-phase it was increased by threefold compared to exponentially growing cells (compare cells sampled at hours 3 and 60). The increased level of transcription from the *rulAB* promoter in stationary-phase indicates that the pWW0-encoded DNA pol V homologue may be induced in bacteria suffering from prolonged starvation to facilitate their adaptation to new growth conditions.

2.2.3. The *rulAB* genes from pWW0 confer growth advantage in stationary-phase phenotype

The presence of plasmids carrying different degradative pathways can be beneficial for bacteria under nutrient limitation conditions. Although these plasmids provide bacteria the ability to grow on a wider spectrum of substrates than those lacking a catabolic plasmid, the available nutrients are quickly consumed and the population enters the stationary-phase. At the onset of starvation there is a selective pressure on mutations that confer a selective advantage (See the section "Growth advantage in stationary phase" in the literature review of this thesis). The occurrence of these mutations in *E. coli* depends on the presence of specialized DNA polymerases. Deficiency of either DNA pol II, pol IV or pol V results in loss of the GASP phenotype (Yeiser *et al*, 2002). So far occurrence of GASP mutations in *P. putida* has been shown only under conditions of phosphate starvation (Eberl *et al*, 1996). We were interested to study whether the pWW0 derived *rulAB* genes could influence the occurrence of the GASP phenotype in *P. putida* and thereby enhance evolutionary fitness of bacteria harboring the plasmid.

We marked the *P. putida* strains used for GASP experiments with different antibiotic resistance markers (resistance to kanamycin or tetracycline) to be able to later separately monitor the number of viable cells (CFU) of 'aged' and 'young' strains in co-culture. The antibiotic resistance genes were inserted into the chromosomally-located transposon Tn*4652*. In GASP experiment cells from an 8-day-old LB culture were diluted at the 1:1000 vol/vol ratio into a fresh (1-day-old) LB culture. The dynamics of both subpopulations in co-culture were monitored for the next 30 days by plating cells onto kanamycin and tetracycline plates to measure separately the number of cells from 'old' and 'young' subpopulations. The occurrence of the GASP phenotype became evident when the relative proportion of cells derived from aged 8-day-old subpopulation exceeded by more than tenfold the number of cells of the young 1-day-old subpopulation (mild GASP) or completely outcompeted the young subpopulation (strong GASP) for 30 days.

We monitored the relative fitness of aged WT strain to young WT, aged PaWrulAB to young PaWrulAB, aged WT to young PaWrulAB and *vice versa*.

Nine parallel competition experiments were carried out with each pair. The dynamics of CFU in subpopulations diverged remarkably during the co-evolution. The difference was observed not only in the mixtures of different combinations of subpopulations but also in parallel experiments initiated with the same pairs of subpopulations started from the same cultures. This indicates that different GASP mutations might be encountered during starvation and that some populations may contain additional mutations having a reduced effect on fitness. Representative examples of GASP competition phenotypes which appeared during the co-cultivation of different subpopulations are shown in Figure 22; Reference IV Figure 3 and data about the co-evolution of the mixed populations is summarized in Table 6; Reference IV, Table 2.

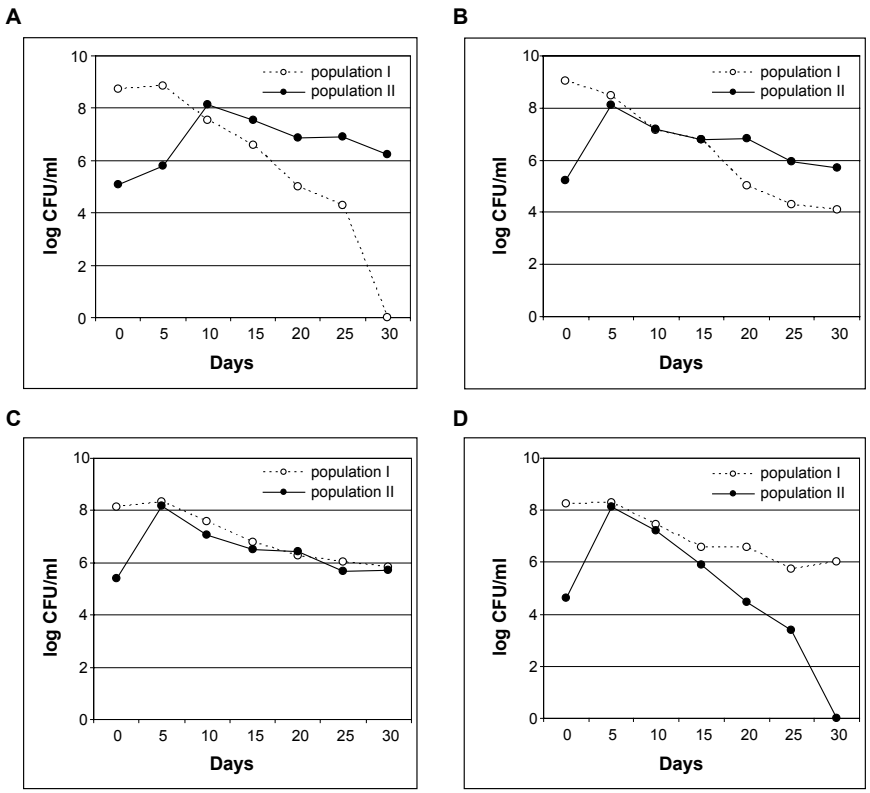


Figure 22. Representative examples of GASP competition phenotypes which appeared during the co-cultivation of aged and non-aged subpopulations of *P. putida*. For simplification, the competing subpopulations are marked as population I and population II. Different expression levels of the GASP phenotype are shown for population I, which consists of cells derived from a 1-day-old (non-aged) culture, and for population II, derived from an 8-day-old (aged) culture. Population II was able to out-compete population I either completely (strong GASP phenotype, panel A) or partially (mild GASP phenotype, panel B) or displayed an equal fitness to that of population I (equal GASP phenotype, panel C). In some cases, population II was either partially or completely out-competed by population I (no GASP phenotype, panel D).

Table 6. The effect of *rulAB* genes on expression of GASP phenotype in *P. putida*

Subpopulation I aged for 8 days	Subpopulation II aged for 1 day	Occurrence of GASP phenotype[a] (from 9 in total)			
		Strong	Mild	Equal	None
WT	**WT**	0	8	0	1
PaWrulAB	**WT**	7	2	0	0
WT	**PaWrulAB**	0	0	2	7
PaWrulAB	**PaWrulAB**	2	5	0	2

[a]Number of cases when the particular subpopulations expressed the GASP phenotype in nine parallel competition experiments if cells derived from 8-day-old and 1-day-old cultures were allowed to co-evolve for 30 days in stationary-phase cultures. Representative examples of expression of the strong, mild, and equal GASP phenotypes or the lack of expression of the GASP phenotype are illustrated in Figure 22.

In all cases studied, during the first five days of the experiment the titer of aged cells increased, becoming equal to the number of cells of the young subpopulation. Later the dynamics of CFU of subpopulations diverged, becoming remarkably affected by the presence or absence of the *rulAB* genes in bacteria. The co-cultivation of aged and young subpopulations of WT cells revealed only a mild expression of the GASP phenotype: the relative proportion of cells derived from aged cultures was increased about five to ten times, with a concomitant decrease in the number of cells of the young subpopulation. Aged PaWrulAB cells expressed the strong GASP phenotype in seven competition experiments, completely outcompeting the young WT subpopulation, and in two experiments the milder expression of GASP phenotype became apparent. Interestingly, in most cases young PaWrulAB cells were also able to completely or partially outcompete the aged WT subpopulations. Only in two cases both subpopulations displayed equal fitness. It seems unlikely that the one-day-old population of PaWrulAB already contained GASP mutants as these mutations are not advantageous in exponentially growing culture. Possibly the three orders of magnitude larger population of PaWrulAB has higher probability of accumulation of beneficial mutations than the WT subpopulation during a week of starvation. We also studied the competition between aged and young PaWrulAB cells. Compared to WT subpopulations, the *rulAB*-carrying cells expressed a stronger GASP phenotype. Taken together our experiments indicate that the *rulAB* genes from TOL plasmid pWW0 increase the probability of *P. putida* cells to accumulate beneficial mutations that allow appearance of the GASP phenotype.

2.2.4. Role of RulAB in stationary-phase mutagenesis (Reference III)

The results shown in the previous section indicate that the *rulAB* genes on the TOL plasmid increase the probability of accumulation of beneficial mutations in *P. putida* cells, allowing genetic adaptation of bacterial populations under environmental stress conditions. DNA polymerase V 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 also in starving populations of *E. coli* (Bhamre *et al*, 2001; Yeiser *et al*, 2002).

These processes occur in stationary-phase cells in which oxidative DNA damage is abundant. The formation of 7,8-dihydro-8-oxo-2′-deoxyguanine (8-oxoG) can give rise to stationary-phase mutations in *E. coli* (Bridges, 1993; Bridges, 1996). 8-oxoG is not generally considered to be a replication-blocking lesion (Bjelland & Seeberg, 2003), but it is highly susceptible to further oxidation and yields a variety of additional products, some of which block DNA replication by replicative DNA polymerase (Neeley & Essigmann, 2006). Studies by Neeley *et al*, suggest a major role for DNA pol V and minor roles for other specialized DNA polymerases in guanine oxidation mutagenesis in *E. coli*. Pol V for instance is responsible for generation of G·C-to-C·G transversions as a result of TLS over 8-oxoG (Timms *et al*, 1999). Other oxidatively damaged nucleotides, such as oxidized adenine are also mutagenic (Kamiya, 2003). However, these types of damage have received less attention so far. The TOL plasmid pWW0 has been shown to confer resistance to reactive oxygen species (ROS) (Yano, 1981). In the light of the observations on *E. coli* DNA pol V, it is possible that the resistance is conferred by the *rulAB* genes and that this resistance increases the survival rate of bacteria during prolonged starvation when oxidative damage accumulates.

We decided to study the involvement of RulAB in stationary-phase mutagenesis and the possible relationship between the mutagenesis and oxidative damage of DNA in starving *P. putida*. In order to assess direct effects due to oxidized damage we generated strains deficient in oxidized guanine repair (GO repair). GO repair involves three enzymes in *E. coli*: MutY and MutM glycosylases and pyrophosphohydrolase MutT (Michaels & Miller, 1992). MutY is known to remove adenine from GO: A mispairs, MutM removes GO from the DNA and MutT hydrolyses GO in the free nucleotide pool to prevent its *de novo* insertion in DNA during replication. We constructed *P. putida* strains deficient in MutY, MutM and MutT. From ten genes encoding putative MutT family (also known as Nudix family hydrolases) proteins we disrupted the one most similar to MutT from *P. aeruginosa*, PP1348, which has been shown to complement *E. coli* MutT deficiency (Oliver *et al*., 2002).

To our surprise the presence of *rulAB* genes does not significantly affect the accumulation rate of stationary-phase mutations in *P. putida* (Figure 23; Reference III, Figures 1 and 2). However, DNA sequence analysis of Phe^+ reversion

mutants accumulated in the presence or absence of the *rulAB* genes in the bacterial chromosome reveales significant differences in spectrum of mutations (Table 7; Reference III, Table 3). The presence of the *rulAB* genes in the bacterial chromosome enhances the replacement of adenine with guanine or cytosine. The proportion of A→ G transitions increases from 6% in the WT strain to 27% ($P < 0.001$) and the proportion of A→C changes from no occurrences in WT strain among 213 mutants analyzed to 2% in RulAB-proficient strain ($P = 0.03$). Unexpectedly, we did not see the increase in the proportion of G-to-C substitutions, mutations that are considered 'DNA pol V fingerprint' in starved *E. coli*. These data demonstrate that although there is no difference in mutation frequency between the *rulAB*-deficient and proficient strains, the *rulAB*-encoded DNA pol V certainly contributes to the occurrence of base substitution mutations in stationary-phase populations of *P. putida*. Analysis of the spectrum of Phe$^+$ revertants indicates that RulAB carries out error-prone synthesis preferably on adenines in DNA. As adenine oxidation products have been found in stationary-phase cells, it is likely that RulAB carries out TLS across these type of damage. What is the role of DNA pol V in TLS across oxidatively damaged adenine products, needs further studies.

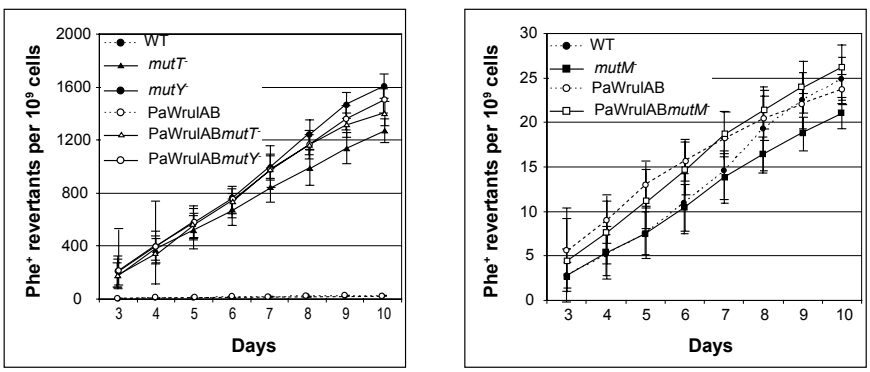


Figure 23. Accumulation of Phe$^+$ revertants on phenol-minimal plates in *P. putida* WT strain and in its RulAB proficient derivatives and GO repair-deficient derivatives: *mutY*$^-$, *mutT*$^-$, and *mutM*$^-$. (A) Accumulation of stationary-phase mutations in strains WT, *mutY*$^-$, *mutT*$^-$, PaWrulAB, PaWrulAB*mutY*$^-$ and PaWrulAB*mutT*$^-$. (B) Accumulation of stationary-phase mutations in strains WT, *mutM*$^-$, PaWrulAB and PaWrulAB*mutM*$^-$. About 2×10^7 cells of *mutY*$^-$, *mutT*$^-$strains carrying the plasmid pKTpheA22TAG with 5×10^8 scavenger cells (WT cells carrying pKT240) or 5×10^8 cells of WT, PaWrulAB and *mutM*$^-$ strain 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 7. Reversion of nonsense mutation (TAG) in Phe$^+$ mutants

Target[a]	mutation	Occurrences							
		No RulAB				RulAB in chromosome			
		WT	***mutM***	***mutT***	***mutY***	**WT**	***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).

Introducing RulAB into *P. putida* strains either deficient in MutY, MutT or MutM does not result in significant changes in mutation frequency compared to the same strains lacking *rulAB* genes (Figure 23; Reference III, Figures 1 and 2). *mutY*$^-$ and PaWrulAB*mutY*$^-$ exhibited on average 100-fold and *mutT*$^-$ and PaWrulAB*mutT*$^-$ 75-fold increase in mutation frequency, respectively, compared to WT during ten days of starvation. The mutation frequency in MutM-deficient strains remained similar to WT (Figure 23, Reference III, Figure 1). When analyzing the mutation spectra we detected some differences: in MutM-deficient background the presence of RulAB increased the occurrence of A→C transversions from 2% to 8% (P = 0.029), and similar change was earlier observed also in WT background (Reference III Table 3). No other differences could be detected in GO-deficient strains in the presence or absence of RulAB. Likely, these differences can be missed due to the enormous increase in A→C and G→T mutations in MutT- and MutY-deficient strains due to the lack of GO repair.

In conclusion, although the DNA pol V homologue RulAB increases fitness of *P. putida* in stationary-phase cultures, it does not result in increased mutation frequency. Our results do not allow to specify the role of RulAB in mutagenesis with regard to oxidative damage caused by oxidatively damaged guanine. The A→C and A→G base substitutions observed in RulAB-proficient strain may be a consequence of the presence of oxidized adenine on DNA, but a conclusive answer to this question awaits further experiments.

CONCLUSIONS

Upon being confronted with DNA damage, bacteria, as any other cell, have two options for survival: either to repair the DNA or to tolerate the damage. If neither of the two succeeds and the lesions remain in the DNA, the cell dies. There are several partially redundant repair pathways to cope with damage, as well as several specialized DNA polymerases to carry out replication across lesions in DNA. In general, DNA repair and damage tolerance due to the action of specialized DNA polymerases are temporally separated processes. First, repair of damage, an intrinsically error-free process is initiated. If damage is not repaired within a certain time, the specialized, often error-prone DNA polymerases are allowed to save the cell from the worst. The best and probably most studied example of these 'sequential' repair and tolerance is the way bacteria deal with UV-induced DNA damage.

I have here addressed the influence of mechanisms of DNA repair and tolerance on mutational processes in bacteria under conditions of carbon starvation. The energy status of bacteria in these conditions is low and chromosome replication and bacterial division are strongly reduced. I have investigated *Pseudomonas putida* pathways that deal with UV-induced damage: nucleotide excision repair and the activity of specialized DNA polymerases.

The main conclusions of my thesis are as follows:

1. Nucleotide excision repair which provides protection from UV-induced DNA damage as well as a vast variety of other types of DNA damage is an important source of stationary-phase mutations in starving cells of *P. putida*. Given that chromosome replication is diminished in stationary-phase, repair synthesis may be the only kind of replication occurring in these conditions. Therefore not only nucleotide excision repair, but also other repair pathways that involve repair synthesis may provide the means for genetic variability that aid adaptation and, on larger scales, evolution.
2. DnaE2, a homologue of the catalytic subunit of replicative DNA polymerase responsible for UV-induced mutagenesis in several bacterial species strikingly reduces the occurrence of UV-induced mutations in *P. putida*. DnaE2 has a similar antimutator effect in cells not exposed to damage.
3. The DNA polymerase V homologue encoded on the toluene degradation plasmid pWW0 is capable of error-prone translesion synthesis across UV-induced lesions. It thus increases the tolerance of bacteria to UV radiation while increasing the mutation frequency. The DNA pol V homologue does not affect the overall mutation frequency in stationary-phase populations, but causes changes in the spectrum of mutations. Moreover, it increases the probability of occurrence of mutations that yield growth advantage in stationary-phase.

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SUMMARY IN ESTONIAN

DNA kahjustuste parandamis- ja talumismehhanismide osalus nälgivates bakterirakkudes toimuvates mutatsiooniprotsessides

Looduses elavad bakterid pidevalt muutuvates keskkonnatingimustes, kus nende kasv on pärsitud toitainete vähesuse tõttu ning bakterite vahel toimub pidev konkurents olemasolevate toitainete pärast. Sellistes stressitingimustes toimub bakterite kohastumine muutlike keskkonna oludega. Lisaks füsioloogilistele ümberkorraldustele toimub bakteritel ka geneetiline kohastumine: tõuseb mutatsioonide tekkesagedus, mis võimaldab suurendada populatsiooni geneetilist mitmekesisust. Mutatsioone, mis tekivad stressioludes nimetatakse statsionaarse faasi mutatsioonideks või ka stressist indutseeritud mutatsioonideks. Stressitingimustes toimuv mutatsioonisageduse tõus arvatakse olevat peamiselt spetsialiseeritud DNA polümeraaside poolt läbiviidava vigaderohke DNA sünteesi osakaalu suurenemise ja replikatsioonijärgse DNA parandamise efektiivsuse languse tagajärg. Samas ei ole ilmselt ühest rada statsionaarse faasi mutatsioonide tekkeks.

Mutatsioonsagedust suurendavad ka mitmete eksogeensete ja endogeensete tegurite toimel tekkinud DNA kahjustused. Rakkudes on DNA kahjustuste eemaldamiseks mitmeid mehhanisme. On DNA parandamise radasid, mis eemaldavad DNA ahelast vaid ühte kindlat tüüpi kahjustusi, kuid ka neid, mille kahjustuse äratundmise spetsiifika on lai. Lisaks DNA kahjustuste parandamisele on rakkudes ka mehhanisme, mis võimaldavad DNA kahjustuste parandamist edasi lükata ehk kahjustusi taluda. Peamiseks selliseks mehhanismiks on spetsialiseeritud DNA polümeraaside võime jätkata DNA sünteesi ka üle DNA kahjustust sisaldava koha (ingl. keeles *translesion DNA synthesis*). Nende DNA polümeraaside poolt läbiviidav DNA süntees on sageli vigaderohke.

Rakkudes on olemas mitmed mehhanisme, mis kaitsevad neid samatüüpi DNA kahjustuste eest. Näiteks on bakterites enamasti kaks alternatiivset DNA parandamisrada, nukleotiidi väljalõike reparatsioon ja ensümaatiline fotoreaktivatsioon, mille peamiseks funktsiooniks peetakse UV-kiirguse poolt põhjustatud kahjustuste eemaldamist. Lisaks nendele on olemas spetsialiseeritud DNA polümeraasid, mis viivad läbi DNA sünteesi üle UV-kiirguse poolt põhjustatud pürimidiin-dimeeride või fotoproduktide. Replikatiivne DNA polümeraas ei ole võimeline üle selliste DNA kahjustuste sünteesi läbi viima. Kaitse UV-kiirguse poolt põhjustatud DNA kahjustuste eest ei pruugi olla nende mehhanismide peamine või ainuke funktsioon kuna paljud, isegi füllosfääris elavad bakterid ei puutu sageli otsese päikesevalgusega kokku. Samas leidub keskkonnas DNAd kahjustavaid kemikaale, mis on kas taimset päritolu või toodetud teiste bakterite poolt, et võõrliikide kasvu pärssida. Kuna bakterid viibivad looduses pidevates stressitingimustes, siis on nendes rakkudes suurenenud ka endogeensete kahjustuste hulk.

Oma töös olen uurinud, kuidas mõjutavad mehhanismid, mille peamiseks funktsiooniks on raku kaitsmine UV-kiirguse mõjul tekkinud DNA kahjustuste

eest, mutatsiooniprotsesse nälgivates bakterirakkudes. Mudelorganismina kasutab meie uurimisrühm mullabakterit *Pseudomonas putida*. Pseudomonaadid on looduses laialt levinud ning kohastunud eluga nii veekogudes, mullas, füllosfääris ning ka taime- ja loomakudedes. Tänu muutlikule elukeskkonnale on pseudomonaadid heaks mudeliks mutatsiooniprotsesside ning kohastumiseks vajalike protsesside uurimiseks.

Töö tulemused võib kokku võtta järgnevalt:

1. Nukleotiidi väljalõike reparatsioon, mis eemaldab lisaks UV-kiirguse poolt põhjustatud DNA kahjustustele veel paljusid teisi DNA struktuuri muutvaid DNA kahjustusi, soodustab nälgivates *P. putida* rakkudes mutatsioonide teket. Kuna nälgivates rakkudes toimuvad kromosoomi replikatsioon ja rakujagunemine harva, siis võib moodustada DNA parandamisega kaasnev replikatsioon suure osa sellistes tingimustes toimuvast DNA sünteesist. Seega võivad nukleotiidi väljalõike reparatsioon ja ka teised reparatsiooni rajad, mille käigus toimub DNA süntees, olla keskkonna tingimustega kohanemiseks vajalike mutatsioonide allikaks.
2. DnaE2, replikatiivse DNA polümeraasi katalüütilise subühiku homoloog, mis mitmetes bakterites põhjustab UV-kiirguse järgset mutatsioonisageduse tõusu, vähendab mutatsioonide teket UV-kiirgusele eksponeeritud *P. putida* rakkudes. Sarnane antimutaator-efekt ilmnes DnaE2-l ka nälgivates *P. putida* rakkudes toimuvate mutatsiooniprotsesside puhul.
3. Laia peremeesringi plasmiidi pWW0 poolt kodeeritud DNA polümeraasi V homoloog RulAB viib UV-kiirgusest põhjustatud kahjustuste olemasolul läbi vigaderohket DNA sünteesi. RulAB suurendab bakterite UV-kiirguse taluvust, põhjustades mutatsioonide tekkesageduse tõusu. Nälgivates *P. putida* rakkudes muudab DNA polümeraasi V olemasolu tekkivate mutatsioonide spektrit ning võimaldab bakteripopulatsiooni kiiremat kohastumist tänu mutantide tekkele, kes on võimelised paremini omastama surnud rakkudest vabanevaid toitaineid.

Tuginedes olemasolevale kirjandusele ja käesoleva töö tulemustele võib välja tuua järgneva hüpoteesi DNA kahjustuste parandamise ja talumise mutageensuse kohta sõltuvalt bakterirakkude seisundist:

(1) Kasvavates rakkudes toimuvad DNA replikatsioon ja rakujagunemine sagedasti ning endogeensete DNA kahjustuste hulk on madal. Nendes tingimustes on spetsialiseeritud DNA polümeraasid ekspresseeritud madalal basaalsel tasemel ning osalevad replikatsioonil harva. Olemasolevad DNA kahjustused parandatakse ning parandamisjärgsel DNA sünteesil osalevad replikatiivsed DNA polümeraasid, mille poolt läbiviidud replikatsioonil tekib vigu madala sagedusega.

(2) DNA kahjustuste olemasolul, näiteks peale rakkude eksponeerimist UV-kiirgusele, eemaldatakse tekkinud kahjustusi esmalt DNA reparatsiooni abil. DNA kahjustuste olemasolul suureneb rakkudes spetsialiseeritud DNA polümeraaside ekspressioon. Spetsialiseeritud DNA polümeraasid viivad läbi potentsiaalselt vigutegevat, üle DNA kahjustuste sünteesi ning võivad osaleda ka DNA parandamisega kaasneval DNA sünteesil, suuren-

dades sel viisil mutatsioonisagedust. Mutatsioonisageduse tõus sellistes tingimustes sõltub olemasolevatest spetsialiseeritud DNA polümeraasidest, nende replikatsioonitäpsusest ning ka kahjustuse tüübist ja hulgast. Juhul kui spetsialiseeritud DNA polümeraas on jätkanud replikatsiooni üle DNA kahjustuse ning sisestanud ahelasse 'vale' nukleotiidi, viib DNA kahjustuse parandamine nukleotiidi väljalõike reparatsiooni rajas mutatsiooni fikseerimiseni.

(3) Stressitingimustes viibivates rakkudes toimuvad kromosoomi replikatsioon ja rakujagunemine madala sagedusega ning endogeensete DNA kahjustuste hulk neis on kõrge. Sellistes tingimustes on spetsialiseeritud DNA polümeraasid ekspresseeritud kõrgel tasemel. Need DNA polümeraasid konkureerivad DNA parandamisega kaasneval DNA sünteesil replikatiivse DNA polümeraasiga ning viies läbi vigutegevat DNA sünteesi, muudavad nad DNA parandamisprotsessi mutageenseks.

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PUBLICATIONS

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II Scientific activities:

1. Main research interests:

1) Genome stability in *Pseudomonas putida* in stress: role of DNA repair and error-prone DNA polymerases
2) Physics of eukaryotic genome organization

2. List of publications:

1) Tarassova K, Tegova R, Tover A, Teras R, Tark M, Saumaa S & Kivisaar M (2009) Accumulation of reactive oxygen species causes mutator phenotype in survival population of carbon-starved rpoS-deficient *Pseudomonas putida*. *J Bacteriol.* **191**,3604–14.
2) Tark M, Tover A, Koorits L, Tegova R & Kivisaar M (2008) Dual role of NER in mutagenesis in *Pseudomonas putida*. *DNA Repair* **1**, 20–30.

3) Saumaa S, Tover A, Tark M, Tegova R & Kivisaar M (2007) Oxidative DNA damage defense systems in avoidance of stationary-phase mutagenesis in *Pseudomonas putida*. *J Bacteriol* **189**, 5504–5514.
4) Koorits L, Tegova R, Tark M, Tarassova K, Tover A & Kivisaar M (2007) Study of involvement of ImuB and DnaE2 in stationary-phase mutagenesis in *Pseudomonas putida*. *DNA Repair* **6**, 863–8.
5) Saumaa S, Tarassova K, Tark M, Tover A, Tegova R & Kivisaar M (2006) Involvement of DNA mismatch repair in stationary-phase mutagenesis during prolonged starvation of *Pseudomonas putida. DNA Repair* **5**, 505–514.
6) Tark M, Tover A, Tarassova K, Tegova R, Kivi G, Hõrak R & Kivisaar M (2005) TOL plasmid pWW0-encoded DNA polymerase V homologue confers evolutionary fitness on *Pseudomonas putida* under conditions of environmental stress. *J Bacteriol* **187,** 5203–13.
7) Tegova R, Tover A, Tarassova K, Tark M & Kivisaar M (2004) Involvement of error-prone DNA polymerase IV in stationary-phase mutagenesis in *Pseudomonas putida*. *J. Bacteriol* **186**, 2735–44.

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- EMBO short term fellowship for studies at Leiden Institute of Chemistry, Laboratory of Molecular Genetics Leiden, The Netherlands; January – March 2008.
- SA Archimedes, FEMS, FEBS ja ASM stipends for participation on different international congresses and meetings 2005–2009.

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- Federation of European Microbiological Societies
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II Teaduslik ja arendustegevus

1. Peamised uurimisvaldkonnad

1) DNA reparatsioonimehhanismide ja spetsialiseeritud DNA polümeraaside osalus *Pseudomonas putida* nälgivates rakkudes toimuvates mutatisooni-protsessides;
2) Kromatiini pakkimise dünaamika ja seos geenieksprессiooniga eukarüoot-setes rakkudes.

2. Publikatsioonide loetelu

1) Tarassova K, Tegova R, Tover A, Teras R, Tark M, Saumaa S & Kivisaar M (2009) Accumulation of reactive oxygen species causes mutator phenotype

in survival population of carbon-starved rpoS-deficient *Pseudomonas putida*. *J Bacteriol.* **191**,3604–14.
2) Tark M, Tover A, Koorits L, Tegova R & Kivisaar M (2008) Dual role of NER in mutagenesis in *Pseudomonas putida*. *DNA Repair* **1**, 20–30.
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5) Saumaa S, Tarassova K, Tark M, Tover A, Tegova R & Kivisaar M (2006) Involvement of DNA mismatch repair in stationary-phase mutagenesis during prolonged starvation of *Pseudomonas putida. DNA Repair* **5**, 505–514.
6) Tark M, Tover A, Tarassova K, Tegova R, Kivi G, Hõrak R & Kivisaar M (2005) TOL plasmid pWW0-encoded DNA polymerase V homologue confers evolutionary fitness on *Pseudomonas putida* under conditions of environmental stress. *J Bacteriol* **187,** 5203–13.
7) Tegova R, Tover A, Tarassova K, Tark M & Kivisaar M (2004) Involvement of error-prone DNA polymerase IV in stationary-phase mutagenesis in *Pseudomonas putida*. *J. Bacteriol* **186**, 2735–44.

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