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TATJANA JATSENKO

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I. Jatsenko T, Tover A, Tegova R, Kivisaar, M. (2010). **Molecular characterization of Rif^r mutations in *Pseudomonas aeruginosa* and *Pseudomonas putida*.** Mutat. Res. 683:106–114.

II. Sidorenko J, Jatsenko T, Saumaa S, Teras R, Tark-Dame M, Hõrak R, Kivisaar M. (2011). **Involvement of specialized DNA polymerases Pol II, Pol IV and DnaE2 in DNA replication in the absence of Pol I in *Pseudomonas putida*.** Mutat. Res. 717(1–2):63–77.

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Author's contribution:

Ref. I: Contributed to design of experiments, performed most of the experiments, contributed to manuscript preparation.

Ref. II: Performed experiments with TLS polymerase-deficient strains, prepared part of the figures, contributed to the editing of the manuscript.

Ref. III: Designed and performed all experiments, constructed most of the strains, prepared figures, carried out statistical analysis and wrote the manuscript.

ABBREVIATIONS

4NQO	4-nitroquinilone 1-oxide
8-OH-dG	8-hydroxy-2' -deoxyguanosine
aa	amino acid
AP	apurinic/apyrimidinic site
BER	Base Excision Repair
bp	base pair
CBM	Clamp-Binding Motif
CFU	Colony-Forming Units
DA	Damage Avoidance
DDT	DNA Damage Tolerance
dsDNA	double-stranded DNA
dNTP	Deoxynucleoside Triphosphate
rNTP	Ribonucleotide Triphosphate
DSB	Double-Strand Break
HR	Homological Recombination
LF	Little Finger
MA	Mutation Accumulation
MIC	Minimal Inhibitory Concentration
MMC	Mitomycin C
MMR	Mismatch Repair
MMS	Methyl Methanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
N^1meA	N^1-methyladenine
N^3meA	N^3-methyladenine
N^3meC	N^3-methylcytosine
N^7meG	N^7-methylguanine
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End-Joining
nt	nucleotide
O^6meG	O^6-methylguanine
PAD	Polymerase Associated Domain
PCNA	Proliferating Cell Nuclear Antigen
PIP	PCNA Interacting Protein box
PHP	Polymerase-Histidinol-Phosphatase
Pol	DNA polymerase
Pol III HE	DNA polymerase III holoenzyme
Pol III*	DNA polymerase III holoenzyme without β-clamp
PPi	Pyrophosphate
Rif	Rifampicin
Rif^r	Rifampicin resistant
RNAP	RNA polymerase
rNTP	Ribonucleotide Triphosphate

ROS	Reactive Oxygen Species
SAM	S-Adenosylmethionine
SIM	Stress-Induced Mutagenesis
SSB	Single Strand Binding protein
ssDNA	single-stranded DNA
TC-NER	Transcription-Coupled Nucleotide Excision Repair
TLS	Translesion DNA Synthesis
UV	Ultraviolet
W-C	Watson-Crick pairing
WGS	Whole-Genome Sequencing

INTRODUCTION

The existence of life depends on the efficiency and prominence of every organism to keep the genetic information intact and transfer it to offspring. Yet, the integrity of our hereditary material is constantly challenged by both endogenously formed agents that arise during normal cellular metabolism and by various exogenous factors, like ultraviolet light and chemicals that can be found everywhere in the environment. Damage in DNA also affects the process of replication: when replicative polymerases encounter damaged nucleotide during DNA synthesis, the progression of the replication fork can be blocked. To counteract DNA damage, cells not only possess highly efficient and accurate DNA-damage repair pathways, but also damage tolerance mechanisms that allow replication to be completed in the presence of DNA damage. Discovered less than 20 years ago, specialized DNA polymerases have revolutionized our understanding of DNA replication, acquisition of new mutations and stability of the genome. This specialized group of DNA polymerases has evolved to promote replication throughout the damaged template in a process known as translesion DNA synthesis (TLS). Although cognate lesions can be bypassed with surprisingly high accuracy and efficiency, TLS is inherently error-prone process. Therefore, TLS polymerases represent a "double-edged sword" in the organism. They ensure the completion of DNA replication and therefore cell survival in the presence of DNA damage. Due to the intrinsic mutagenic nature, TLS polymerases also introduce genetic diversity that can be evolutionary beneficial for adaptation and survival under changing and stressful conditions. On the other hand, surplus of mutations can have dramatic consequences by increasing the risk of genomic instability. Dysregulation of TLS has been linked to cancer development in human, while in bacteria TLS polymerases are important for the emergence of antibiotic resistance and virulence. In turn, bacterial TLS systems can be a potential target for antimicrobial treatment, so understanding to which extent TLS contributes to genetic diversity and how organisms keep the mutation rates in balance is of great fundamental and translational importance.

Bacteria have a remarkable capacity to maintain genomic stability under constantly changing environmental conditions. Although a lot of research has been done in a model organism *Escherichia coli*, it is extremely important to elucidate mutagenic processes, including function and role of TLS polymerases and TLS, in other bacteria, as many species harbor different set of genes responsible for DNA damage repair and tolerance. Hence, the function, specificity and contribution of TLS to mutagenesis can drastically vary between different studied organisms.

In the research group of prof. Maia Kivisaar, we use *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Pseudomonas putida* (*P. putida*), representatives of the most ubiquitous, diverse and ecologically significant bacterial genera, as model organisms to study mutagenic mechanisms in bacteria. *P. putida,* found

throughout various niches, is able to metabolize a wide range of different compounds, and is therefore important in bioremediation. For human, *P. aeruginosa* is an important and challenging opportunistic pathogen with an overwhelming ability to adapt and develop multidrug resistance through mutational changes and chromosomally encoded resistance mechanisms. The aim of the current thesis was to evaluate the mutagenic potential of specialized DNA polymerases in *P. putida* cells in the absence of exogenous DNA damage and examine their involvement in mutagenic processes in *P. putida* lacking DNA Polymerase I functions. In addition, the importance of TLS polymerases in alkylation damage tolerance in Pseudomonads and factors underlying genomic stability in both species were explored.

I REVIEW OF LITERATURE

1.1 Overview of DNA synthesis and replication

1.1.1 DNA polymerases and process of DNA synthesis

In 1956, a group of scientists led by Arthur Kornberg discovered an enzyme that was able to incorporate nucleotides into DNA and perform polymerization reaction in extracts of *Escherichia coli* (*E. coli*) (Kornberg *et al.*, 1956). Two years later, this enzyme was purified and now it is known as DNA polymerase I (Pol I) (Lehman *et al.*, 1958). Soon after, a second DNA-synthesizing enzyme in *E. coli*, DNA polymerase II (Pol II) was purified (Kornberg and Gefter, 1971). During the time of purification, the third DNA polymerase III (Pol III) was discovered in *E. coli*, which later was found to be a major replicative DNA polymerase, essential for cell survival (Gefter *et al.*, 1972; McHenry, 2011; Vaisman and Woodgate, 2017). Up to date, five DNA polymerases have been described in *E.coli*, but the role of the latest discovered Polymerase IV (Pol IV) and Polymerase V (Pol V) extends beyond the canonical DNA replication.

To date, there are eight families of DNA polymerases that are grouped based on amino acid sequence homology: A, B, C, D (polymerases found only in archaea), X and Y, as well as reverse transcriptases (RT) and recently discovered archaeo-eukaryotic primases (AEP), depicted in Fig. 1 (Ito and Braithwaite, 1991; Ishino *et al.*, 1998; Burgers *et al.*, 2001; Guilliam *et al.*, 2015). Each family fulfils a specific mission in DNA synthesis processes. In general, replicative DNA polymerases, called replicases, are highly accurate enzymes that play a pivotal role in genomic DNA replication. They belong to the B-family in eukaryotes, A- and C- in bacteria, and B- and D-families in archaea (Lujan *et al.*, 2016). Most of the high-fidelity replicases possess exonucleolytic 3′→5′ proofreading activity (Reha-Krantz, 2010). In contrast to replicases, X- and Y-family members are distributive DNA polymerases, lacking proofreading activity, which function in DNA damage repair and tolerance processes, rather faithful chromosomal DNA replication (Ling *et al.*, 2001; Uchiyama *et al.*, 2009). Y-family members represent a unique class of specialized DNA polymerases that mediate replication of damaged DNA (process known as translesion DNA synthesis, TLS) (Ohmori *et al.*, 2001). X-family polymerases are mainly involved in DNA repair pathways, performing gap-filling synthesis associated with base excision repair (BER) and double-strand break (DSB) processing during non-homologous end-joining (NHEJ) pathway and V(D)J recombination (Moon *et al.*, 2007; Yamtich and Sweasy, 2010). RT enzymes synthesize DNA using RNA as a template (Baltimore, 1970; Belfort *et al.*, 2011). Most recently discovered primase-polymerases (PrimPol), an AEP superfamily members, expanded the horizons of enzymes involved in DNA synthesis (García-Gómez *et al.*, 2013; Guilliam *et al.*, 2015). These enzymes, having both primase and polymerase activities, are

able to perform *de novo* DNA synthesis and display unconventional roles in DNA damage tolerance and repair pathways (Guilliam and Doherty, 2017).

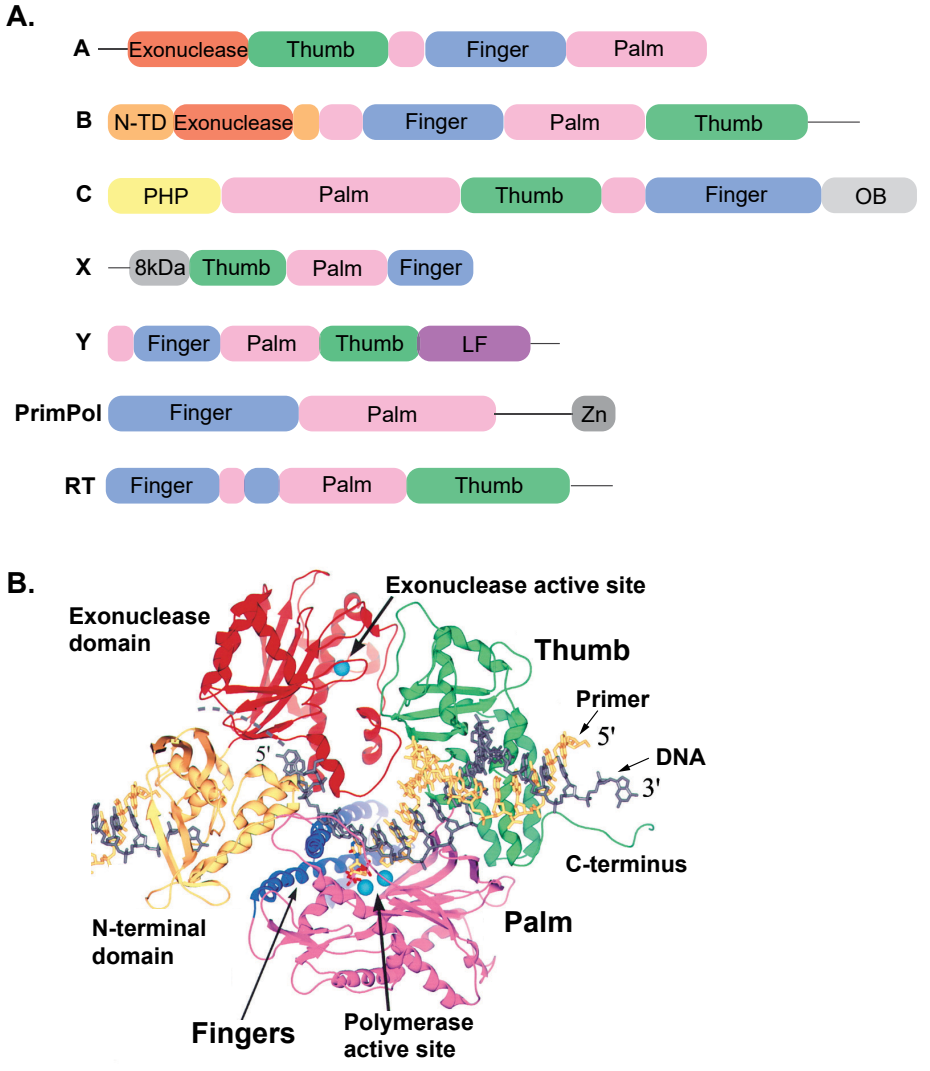

Figure 1. Domains of DNA polymerases and ternary polymerase-DNA-dNTP complex. (A) Domains of DNA family polymerases (Abbreviations of domains: N-TD: N-terminal domain; PHP: polymerase and histidinol phosphatase domain; OB: oligonucleotide binding; LF: Little finger). Based on (Yang and Gao, 2018). (B) Representative of B-family polymerases (RB69 DNA polymerase) is demonstrated, with subdomains indicated as follows: "N-terminal" domain in orange, "exonuclease" in red, "palm" in pink, "fingers" subdomain in blue and "thumb" in green. DNA template is indicated in grey with primer in gold. Two metal ions in the polymerase active site are indicated as blue "spheres". Adapted by permission from (Franklin *et al.*, 2001).

Most of the organisms possess more than one DNA polymerase. For example, at least 17 different DNA polymerases have been purified and characterized in human. However, only five of them mediate high-fidelity genomic (B-family Pol α, Pol δ and Pol ε and telomerase from RT-family) and mitochondrial DNA synthesis (A-family pol γ). Y-family polymerases Pol κ, Pol η, Pol ι, and Rev1, in coordination with B-family member Pol ζ mediate replication across DNA lesions (*i.e.*, mediate TLS). The remaining DNA polymerases also are capable to perform TLS and participate in DNA repair pathways (Waters *et al.*, 2009; Sale, 2013; Yang, 2014; Vaisman and Woodgate, 2017; Zhao and Washington, 2017; Shanbhag *et al.*, 2018). *E. coli,* as was mentioned above, has five different DNA polymerases, each having important functions in DNA replication, repair and damage tolerance (Friedberg, 2005; Sutton, 2010). Pol III and Pol I are high-fidelity enzymes, responsible for the genomic DNA replication (Gefter *et al.*, 1971; Kornberg and Baker, 2006). In addition to replication, Pol I plays important role in nucleotide excision repair (NER) (Moolenaar *et al.*, 2000). The function of the second discovered in *E. coli* polymerase Pol II (*polB*-encoded), member of the B-family, remained for a long time enigmatic. Possessing exonuclease activity, Pol II has high fidelity with an error rate $\leq 10^{-6}$ (Cai *et al.*, 1995), and it is involved in replication restart following ultraviolet (UV)-irradiation (Rangarajan *et al.*, 1999, 2002). Moreover, Pol II, with *dinB*-encoded Pol IV and *umuDC*-encoded Pol V, members of specialized Y-family of polymerases, are capable to replicate damaged DNA, and play important role in damage tolerance and mutagenesis (Tang *et al.*, 1999; Wagner *et al.*, 1999, 2002; Napolitano *et al.*, 2000; Fuchs and Fujii, 2007).

Despite the fact that DNA polymerases have different fidelity, processivity and catalytic specificity, they all share common general structure for polymerase domain and catalytic mechanism of nucleotidyl transferase reaction (Rothwell and Waksman, 2005). Almost all known polymerases structurally resemble "partially open right hand" topology, with functional domains represented as "thumb", "palm" and "finger" subdomains (Fig. 1) (Wu *et al.*, 2014). One exception is the recently discovered PrimPol that lacks traditional "thumb" subdomain (Rechkoblit *et al.*, 2016). In addition, members of the X-family polymerases share "left-handed topology" because of the non-homologous "palm" subdomain (Beard and Wilson, 2000). "Thumb" interacts with a major grove of primer-template DNA duplex and is involved in the positioning and translocation of DNA molecule through polymerase; "finger" subdomain is involved in incoming nucleotide-binding and its proper positioning (Fig. 1B) (Ollis *et al.*, 1985; Federley *et al.*, 2010). The catalytic center is located in the "palm", which is also the most conserved subdomain across all polymerase families (Johansson and Dixon, 2013). It contains two to three catalytically essential conserved amino acids that coordinate two metal ion-mediated reaction mechanism of DNA synthesis (nucleotidyl transfer reaction) (Yang *et al.*, 2006; Johansson and Dixon, 2013). DNA polymerases catalyze the formation of phosphodiester bond between the 3′-OH of the DNA primer and the α-phosphate of the incoming deoxynucleoside triphosphate (dNTP)

(Nakamura *et al.*, 2013). In each catalytic cycle DNA template governs the specificity of the incoming nucleotide via the Watson-Crick (W-C) base pairing (Loeb and Monnat, 2008; Tsai, 2014). Before the completion of catalytic reaction, polymerase-DNA-dNTP complex undergoes a number of conformational transitions that prepare the active site for a chemical step, serving also as critical kinetic fidelity checkpoints (Joyce and Benkovic, 2004; Bermek *et al.*, 2011), which will be described further.

1.1.1.1 Mechanisms of high-fidelity DNA synthesis

DNA polymerases can be characterized as high-fidelity (*e.g.,* in *E. coli* replicative Pol III and Pol I) and low-fidelity DNA polymerases (*e.g.,* representatives of Y-family polymerases Pol IV and Pol V in *E. coli*) (Kunkel, 2009). The fidelity of DNA polymerization mainly depends on the intrinsic ability of polymerase to select correct dNTP during DNA replication, and high-fidelity polymerases have much higher efficiency of active nucleotide selection and insertion in comparison with low-fidelity DNA polymerases (Beard *et al.*, 2002; Joyce and Benkovic, 2004). Such selectivity is achieved during multistep process of DNA polymerization by employing a kinetic and chemical checkpoints to actively select between the right W-C base pairing and discriminate against other dNTPs and ribonucleotide triphosphates (rNTPs) (Johnson and Beese, 2004; McCulloch and Kunkel, 2008; Johnson, 2010). The correct dNTP binding, conformational change and nucleotidyl transfer reaction are the main checkpoints that ensure high-fidelity of replicative polymerases (Franklin *et al.*, 2001; Freudenthal *et al.*, 2013; Liu *et al.*, 2016). Polymerase binding to a primer-template leads to the formation of a binary complex (open conformation). The binding of the correct (complementary) nucleotide promotes a large conformational change of the "finger" subdomain, which moves from an open binary complex to the closed catalytically active ternary complex. Such conformational change forms a tight pocket, leading to the ideal alignment of the phosphate group of the incoming dNTP and 3′-end of the primer, needed for nucleotidyl reaction to occur (Li *et al.*, 1998; Doublié *et al.*, 1999; Kool, 2002; Joyce and Benkovic, 2004). Conformational selection increases the accuracy of replicative polymerases almost 100-fold (Yang and Gao, 2018). Recent single-molecule studies demonstrated that the complementary nucleotide (or correct W-C base pairing) leads to a significant stabilization of the ternary polymerase-DNA-dNTP complex. Otherwise, in the presence of non-complementary dNTP, rNTP or damaged nucleotide, the ternary complex is destabilized (Markiewicz *et al.*, 2012), leading to a partially closed state of "finger" subdomain. In this case, re-opening of the ternary complex occurs much faster, causing the release of the incorrect dNTP (Johnson, 2010; Evans *et al.*, 2015).

Catalytic metal ions represent an important basis for the chemical selection checkpoint, significantly helping the polymerase to attain high fidelity (Yang *et al.*, 2006; Vashishtha *et al.*, 2016; Yang and Gao, 2018). The initial association of

one of the ions is constantly revised, and in the case of a mismatch or damaged dNTP, the incoming nucleotide would be rejected before the second metal ion is recruited to the active site (Yang *et al.*, 2016; Yang and Gao, 2018). Taken together, both conformational and chemical selection account almost for 10^{-4}-10^{-5} to overall fidelity in replicative DNA polymerases (Kunkel, 2009).

In rare cases, when the incorrect nucleotide is incorporated, replicative DNA polymerases switch from DNA polymerization to proofreading mode to excise the misincorporated nucleotide. Almost all known high-fidelity polymerases contain a 3′–5′ exonuclease proofreading activity either built-in on the same polypeptide, *e.g.,* like in *E. coli* Pol I, or a separately interacting subunit in a multienzyme complex, *e.g.,* exonuclease subunit ε of *E. coli* Pol III (Jovin *et al.*, 1969; Maki and Kornberg, 1987; Johansson and Dixon, 2013). Exonuclease activity contributes almost 10-to-100-fold to the net fidelity of polymerase (Kunkel, 2009). When the incorrect nucleotide is added to the 3′ terminus of the primer strand, the catalysis is slowed down and the primer terminus is directed with the help of the polymerase to the exonuclease subdomain for editing (Patel *et al.*, 1991; Xia and Konigsberg, 2014). In the presence of mismatch, the "thumb" subdomain avoids translocation and holds DNA duplex constantly in a minor groove, thus allowing mismatched primer termini to shuttle to and from the exonuclease active site (Ren, 2016). To reach the exonuclease active site, three to four nucleotides from the mismatched terminus have to be separated from the template. One recent study established a new paradigm in the correction of the mismatched nucleotide by high-fidelity polymerases (Fernandez-Leiro *et al.*, 2017). Using replicative *E. coli* Pol III as a model, they demonstrated that the mismatch leads to the distortion of the DNA, which enables the mismatched nucleotide to reach the exonuclease that is located three base pairs away. In this way, the mismatch actually self-corrects, whereas the exonuclease has a passive role in the excision of the terminal nucleotide (Fernandez-Leiro *et al.*, 2017). Similar three base pair distortions have been observed also in the A and B-family polymerases, where the proofreading is located in the same polypeptide as polymerase, suggesting that distortion-induced self-correcting mechanism might be common (Fernandez-Leiro *et al.*, 2017).

High-fidelity DNA polymerases with proofreading activity sense the mismatch not only at the insertion site, but up to 4–5 base pairs from the primer terminus (Carver *et al.*, 1994; Fujii and Fuchs, 2004; Swan *et al.*, 2009). The mispair distorts geometry of the DNA duplex, affecting the hydrogen bond contacts of the polymerase with DNA. As a result, DNA binding to the polymerase active site weakens, triggering relocation of the primer-template termini for proofreading, thus helping to maintain genome integrity (Kennard and Salisbury, 1993; Reha-Krantz, 2010; M. Wang *et al.*, 2011).

Some replicative DNA polymerases also possess additional Polymerase-Histidinol-Phosphatase (PHP) domain (Fig. 1A) with a suggested pyrophosphatase activity, which hydrolyzes the released pyrophosphate (PPi, a byproduct of nucleotidyl reaction), shifting reaction towards polymerization (nucleotide

incorporation) (Aravind and Koonin, 1998). For example, catalytic subunit α of *E. coli* replicative Pol III has an active PHP domain, and alterations in PHP function affect viability of bacteria and also lead to increased mutagenesis. This suggests that relation of DNA polymerization and PPi hydrolysis rates might be an important mechanism ensuring proficient genome replication and genome stability (Lapenta *et al.*, 2016).

Proofreading activity of high-fidelity polymerases not only corrects misincorporations, but also prevents bypass of DNA lesions. Even in cases when nucleotide will be incorporated opposite the DNA lesion, inability to elongate the distorted primer termini or futile insertion-proofreading correction cycles will lead to replicative polymerase stalling (Borden *et al.*, 2002; Pages *et al.*, 2005; Reha-Krantz, 2010). Unlike high-fidelity polymerases, Y-family DNA polymerases catalyze translesion DNA synthesis, perform DNA extension in regions, where high-fidelity DNA polymerases stall, and efficiently overcome traditional W-C base-pairings (Tsai, 2014). TLS DNA polymerases, including the underlying mechanisms of their action, will be discussed in greater detail in Chapter 1.3

1.1.1.2 Process of DNA replication in E. coli

High-fidelity DNA polymerases carry out DNA replication in a context of a large multiprotein assembly, termed replisome (Fig. 2) (Johnson and O'Donnell, 2005). This dynamic complex, consisting of DNA helicase (DnaB), DNA polymerase(s), primase, single-strand DNA binding proteins (SSB) and different scaffolding components, enables bi-directional replication of both leading- and lagging- DNA strands simultaneously (O'Donnell, 2006).

Duplication of a circular chromosome of *E. coli* is initiated from a unique origin, *oriC* (Fig. 2A) (Reyes-Lamothe *et al.*, 2008; Leonard and Méchali, 2013), and proceeded by a pair of independent sister replisomes that move bi-directionally toward the terminus (*ter*) region, located opposite the *oriC* (Fan and Strick, 2015). The assembly of replication machinery is promoted by DnaA proteins that recruit replicative helicase DnaB onto single-stranded DNA (ssDNA) through DnaB-DnaC (helicase loader) complex (Makowska-Grzyska and Kaguni, 2010; Bell and Kaguni, 2013). Loaded DnaB initiates unwinding of the parental DNA duplex while translocating along the lagging-strand in the 5′-3′ direction. Translocating helicase then interacts with the specific RNA polymerase – DnaG primase, responsible for the synthesis of short, approximately 12 base pair (bp) RNA primers (Tougu and Marians, 1996; Frick and Richardson, 2001). This, in turn, triggers the assembly of DNA polymerase III holoenzyme (Pol III HE) multisubunit complex (Fig. 2B). Pol III HE contains two or three replicative polymerase Pol III cores (αεθ), β-clamps (β_2) and a clamp loader complex ($\gamma^{(3-N)}\tau^{N}\delta\delta'\chi\psi$, where N is either 2 or 3, depending on the number of Pol III cores observed in different studies (McInerney *et al.*, 2007; Reyes-Lamothe *et al.*, 2010; Dohrmann *et al.*, 2016)). Pol III core consists of three subunits:

(i) catalytic α subunit, which catalyzes DNA replication, (ii) 3′-5′ proofreading exonuclease subunit ε and (iii) a small θ subunit, which binds tightly to proofreading subunit ε and stabilizes its activity (Scheuermann *et al.*, 1983; Taft-Benz and Schaaper, 2004). Each Pol III core in HE is bound to a sliding β-clamp and to a τ subunit of the clamp loader complex. Clamp loader organizes the Pol III HE into a single complex by associating two cores together with the DnaB helicase at the replication fork. Moreover, clamp loader also assembles ring-shaped β-clamps onto DNA (Kim *et al.*, 1996; Park and O'Donnell, 2009; McHenry, 2011). When β-clamp is loaded, it tethers core polymerase to the primed template and, by sliding behind the polymerase, confers a high degree of processivity and speed (Stukenberg *et al.*, 1991; Georgescu *et al.*, 2012). Such assembled Pol III HE is capable of simultaneous high-speed replication of both leading- and lagging-strands.

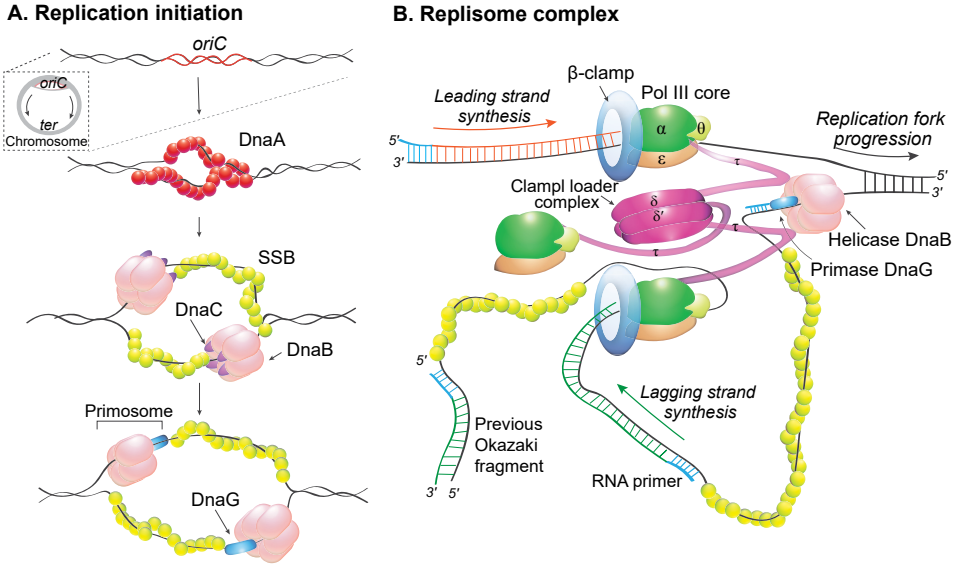

Figure 2. DNA replication in *E. coli.* Replication initiation (A) and a model of replisome complex and DNA replication (B) in *E. coli*. Trimeric replisome model, containing three polymerase cores (Pol III core) associated with three τ copies of the camp loader ($\tau^3\delta\delta'\chi\psi$) is illustrated (McInerney *et al.*, 2007; Reyes-Lamothe *et al.*, 2010). Dimeric replisome models, where only two replicative cores, associated with $\gamma\tau^2\delta\delta'\chi\psi$ clamp loader are also suggested (Dohrmann *et al.*, 2016).

Because of the antiparallel nature of DNA molecule itself, two nascent DNA strands are synthesized via different ways, as the lagging-strand is duplicated in the opposite direction of the replication fork movement (Fig. 2B) (Hamdan *et al.*, 2009). DNA synthesis of the leading-strand on undamaged DNA template is highly processive and is thought to be constant, and requires theoretically only one clamp loading at *oriC*. Replication of the lagging-strand occurs via discontinuous synthesis of short 1000–2000 bp long Okazaki fragments that are joined and processed into a continuous strand later (Lewis *et al.*, 2016). During replication, DnaB helicase movement leads to the formation of ssDNA replication loops between the lagging-strand core and helicase that become coated with SSB proteins, which protect ssDNA from nucleases and remove secondary DNA structures (Yao and O'Donnell, 2008). The synthesis of the lagging-strand requires frequent re-priming, which is mediated by coordinated interplay between helicase and primase, leading to the synthesis of short RNA primers, followed by loading of a new sliding clamp every 2–3 s for each new Okazaki primer (Corn and Berger, 2006; Hayner *et al.*, 2014). Subsequently, Pol III elongates primers and dissociates after the completion of Okazaki fragment to further associate back with the upstream primer. When Okazaki fragment is synthesized, the β-clamp that is left behind first binds Pol I, which removes RNA primers and fills in the processed fragments with dNTPs. Next, Pol I switches its place with DNA ligase that further seals completed Okazaki fragments into continuous DNA molecule (Lopez de Saro and O'Donnell, 2001).

Recent studies demonstrate that there is no strict coordination between the leading- and lagging-strand syntheses, and that the replication process is much more dynamic than was suggested before (Beattie *et al.*, 2017; Graham *et al.*, 2017; Lewis *et al.*, 2017). Single-cell microscope studies demonstrated frequent dissociation of Pol III* (Pol III HE, lacking β-clamp) from the replisome, and exchange with other copies of Pol III* from solution during DNA synthesis, with only DnaB being constantly associated with the DNA, challenging the idea of static replisome assembly (Beattie *et al.*, 2017). Such dynamic exchange was also observed in live cells, representing a potential mechanism that allows replisomes to deal with various DNA obstacles and provide frequent and rapid access of other partners to the replication fork (Beattie *et al.*, 2017; Lewis *et al.*, 2017). Polymerases within the replisome complex can also function independently in time, and such stochastic action of replisome components ensures complete duplication without the need of coordinated leading- and lagging-strand synthesis, challenging the historically suggested coordinated fashion of DNA replication process (Graham *et al.*, 2017). As such, stochastic model of replication, driven by stochastic and independent actions of polymerases with frequent exchanges may exist in *E. coli* cells.

1.2 Fidelity of DNA replication in living cells

"Mutations are genetic fortuities..?"

DNA replication and cell division underlie the foundation of biological inheritance in all living organisms. Errors, introduced during genomic replication, represent an important source of genetic variability, which is also the main driving force of evolution. On the other hand, the majority of new mutations are either neutral or deleterious to fitness (Keightley and Lynch, 2003), and organisms have acquired mechanisms to keep the mutation rate in balance (Drake, 1991; Sniegowski *et al.*, 2000; Denamur and Matic, 2006). Initial evolutionary models suggested that mutations arise stochastically, but nowadays studies suggest the opposite, demonstrating non-random distribution of genetic variation across genomes (Drake, 2007a, 2007b; Parkhomchuk *et al.*, 2009; Hodgkinson and Eyre-Walker, 2011; Martincorena *et al.*, 2012). Therefore, unravelling the mechanisms of mutational processes is important for understanding the maintenance of genomic stability, fundamentals of evolutionary processes, and the origins of cancer in human or antibiotic resistance within pathogens.

1.2.1 Methods for estimating mutation rates

Our understanding of the mutation rate variation across species and its effect on fitness has been restricted by the technical difficulties and reliable approaches that limited research to indirect estimates in model organisms (Drake, 1991; Eyre-Walker and Keightley, 2007; Lynch *et al.*, 2016). However, over the last decade affordability and accessibility of whole-genome sequencing (WGS) strategies allowed us to obtain direct estimates of genome-wide mutation rates and spectra across different genomes, revealing novel and important data (Lee *et al.*, 2012; Foster *et al.*, 2015; Long *et al.*, 2015; Sung *et al.*, 2015; Dettman *et al.*, 2016). There are three commonly used techniques to determine mutation rates in bacteria: (i) fluctuation analysis, (ii) mutation accumulation method (MA), and (iii) comparative genomics (Williams, 2014). In comparative genomics, DNA sequences from organisms diverged at evolutionary time scale are compared to evaluate mutation rates and spectra (Ochman *et al.*, 1999; Hardison, 2003; Ochman, 2003). Fluctuation analysis, a historical workhorse originally described already in 1940s (Luria and Delbrück, 1943), is based on the counts of cells that gain a mutation in a reporter gene (phenotypic marker) (Fig. 3A) (Drake *et al.*, 1991; Foster, 2006). This method is widely applied for large bacterial or viral populations, enabling detection of low mutation rates (Foster, 2006). Different specific mutation assays have been developed, varying in a method of selection, number and type of detectable mutation events. Mutation assays can be classified into two groups: forward and reverse (or reversion) mutation assays (Schroeder *et al.*, 2017). Forward mutation reporter assays are based on inactivation of a reporter gene leading to loss-of-function of the enzyme.

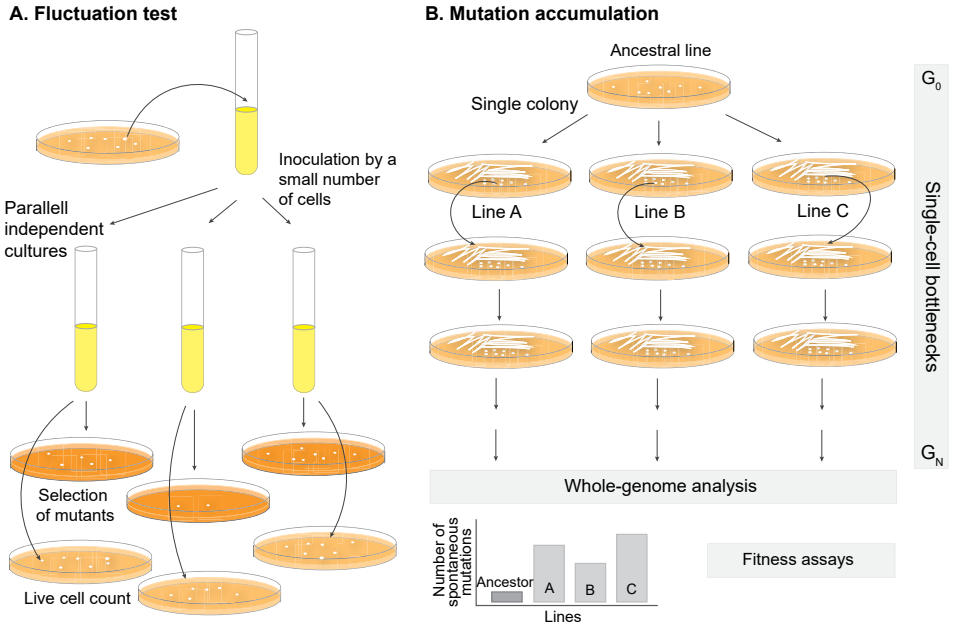

Figure 3. Methods for estimation of spontaneous mutations. Luria-Delbrück fluctuation test (A) and mutation accumulation method (MA) (B). **A.** In Luria-Delbrück fluctuation test, a small number of cells (mutant free inocula) is inoculated into a large number of parallel individual liquid cultures. The cultures are incubated for a period of time in the absence of selection to allow mutations spontaneously arise. At the end of incubation period, selection is applied: each culture is transferred onto a selective medium that allows only mutants to survive or grow and form a colony (Luria and Delbrück, 1943). To estimate the total number of cells in a culture, appropriate dilutions of a few cultures are plated onto nonselective media. The measure of distribution of the numbers of mutants among the number of parallel cultures enables evaluation of mutation rates. This way, fluctuation test enables to determine the probability of mutation events per cell per division (generation) (Rosche and Foster, 2000; Foster, 2006). **B.** In MA experiment, individual mutation accumulation lines are derived from the genetically uniform ancestral population and allowed to accumulate mutations over generations (Eyre-Walker and Keightley, 2007). To establish individual parallel lineages, an ancestral population is plated onto agar plates to retrieve single colonies. Individually picked single colonies are then randomly chosen and passaged through generations. This way, the individual MA lineages are put through a number of repeated bottlenecks, allowing all nonlethal mutations to accumulate over time. After the period of mutation accumulation, a single colony from each lineage is randomly picked and analysed in parallel with ancestral population. Mutation rate is then estimated by knowing the number of generations of MA line and number of mutations identified by sequencing (Trindade *et al.*, 2010; Foster *et al.*, 2013; Singhal, 2017).

In contrast, reverse assays are based on a functional rescue of a reporter gene (gain-of-function) upon mutation, allowing the growth of revertants under selective conditions (Skopek *et al.*, 1978; Standley *et al.*, 2017). The example of a widely used forward mutation assay is based on the inactivation of a *lacI* gene that encodes a repressor of the *lac* operon. Spontaneous loss-of-function mutations in the *lacI* gene disables the repression of the *lac* operon, permitting its constitutive expression and enabling selection of mutants on medium containing phenyl-*β*-D-galactoside sugar as sole carbon source (Gordon *et al.*, 1988; Schaaper and Dunn, 1991; Swerdlow and Schaaper, 2014). Another commonly used system to study mutations by fluctuation assay is based on the counts of bacteria that gain antibiotic resistance (so-called "reversion" mutations in chromosomally encoded genes that confer antibiotic resistance) (Pope *et al.*, 2008; Schroeder *et al.*, 2017). For instance, one commonly used antimicrobial, rifampicin (Rif), targets *rpoB*-encoded β subunit of RNA polymerase (RNAP). Mutations in the *rpoB* gene, affecting the RNAP β subunit, result in antibiotic resistance, enabling bacteria to grow in the presence of antimicrobial (Garibyan *et al.*, 2003; Goldstein, 2014). The β subunit of RNAP is highly conserved across prokaryotes, as such, the use of the *rpoB* gene as a mutation reporter gene represents a simple and robust method for the estimation of mutation rates and spectra in different bacteria species (Campbell *et al.*, 2001; Garibyan *et al.*, 2003).

The above described assays, in turn, can be divided based on the mutant selection method: lethal, when non-mutants cannot survive under selective conditions or non-lethal (growth limiting), when all cells survive, but only mutants with the mutation in the reporter gene can grow and form colonies. Under lethal conditions (*e.g.,* antibiotic selection), only mutations that are formed during the growth phase (in dividing cells) can be detected. In non-lethal conditions, also mutations that occur during prolonged incubation can be studied (Godoy *et al.*, 2017). The most known example of such test-system is the developed *E. coli* FC40 strain, which is unable to utilize lactose (Lac^-) as a sole carbon source, because of a frameshift mutation in a *lacI* gene (Cairns and Foster, 1991). Plated onto lactose-minimal plates Lac^+ revertants, which appear on the second day, represent the mutations that occurred under non-selective conditions in a liquid culture during exponential growth. However, Lac^+ mutants that continue to appear on minimal plates further represent the processes that occur during non-lethal selection in the absence of cell division, and are known as stationary phase or adaptive mutations (Cairns *et al.*, 1988; Cairns and Foster, 1991).

Popularity growing mutation accumulation method (MA) uses ongoing advances of WGS and provides direct measures of mutation rates and spectra across the entire genome accumulated in the absence of selection (Fig. 3B) (Lynch *et al.*, 2008, 2016; Halligan and Keightley, 2009). The combination of MA with WGS has enabled researches to determine the number, identity and location of both single nucleotide events, as well as large-scale genomic events. Such approach made it possible to reveal some of the underlying bases of mutagenesis, and comparisons with the ancestral strain allowed direct estimation of

the mutation rates across the genomes of different species (Lee *et al.*, 2012; Sung *et al.*, 2012; Keightley *et al.*, 2014; Zhu *et al.*, 2014; Foster *et al.*, 2015; Dettman *et al.*, 2016). In addition, MA-WGS experiments paired with fitness evaluation of MA lines offered the unique opportunity to study directly the effect of spontaneous mutations on fitness of the organism, revealing how new mutations and their selection can lead to adaptation and evolutionary change (Dillon and Cooper, 2016; Kraemer *et al.*, 2017).

1.2.1.1 What is the actual mutation rate in E. coli?

The spontaneous mutation rates estimated by different approaches using distinct reporter systems can vary greatly. As an example, in most studied organism *E. coli* the spontaneous mutation rate estimated by the analysis of reporter loci *lacI* was 7.9×10^{-10} per nucleotide (Drake *et al.*, 1991; Drake, 2009). Substitution rate estimated by comparative genomics, estimated by the 16S rRNA gene sequence divergence, was almost one magnitude lower than Drake's estimate (Ochman *et al.*, 1999). If scored for rifampicin resistance, the mutation rate was 0.33×10^{-10} mutations per nucleotide per generation (Lee *et al.*, 2012). The mutation rate estimated by WGS of *E. coli* MA lines was only third of that estimated by Drake, being 1.99×10^{-10} per nucleotide per generation (Lee *et al.*, 2012). The differences in the estimates of mutation rates derived from the analysis of MA lines and specific reporter loci is not surprising, as differences in protocols and growth conditions may affect the appearance and selection against the specific mutation type. The biggest issue associated with the investigation of mutational processes relying on reporter genes is that reporters may not be a representative of the whole genome, and the number of detectable mutations can be restricted sometimes only to a few nucleotides of the small reporter loci (Foster *et al.*, 2015). On the other hand, despite the fact, that WGS approaches can give the overview of the mutational load across the whole genomes, they uncover only single mutational events (Lee *et al.*, 2012). In addition, they are more laborious, and therefore not very applicable for the investigation of hotspot mutations at single nucleotide level. As such, combination of reporter loci studies with new WGS approaches will probably be beneficial in understanding of mutational processes and underlying mechanisms (Lee *et al.*, 2012; Williams, 2014).

One fascinating study has recently investigated the dynamics of spontaneous mutations and their effect on fitness in single *E. coli* cells directly in real time (Robert *et al.*, 2018). By using microfluidic devise combined with time-lapse imaging and fluorescently tagged mismatch repair protein MutL that binds at the sites of replication errors, the group of scientist has developed and performed microfluidic MA experiment (μMA) and mutation visualisation experiments. Over the three-day experiment, corresponding to app. 200 generations, they estimated that spontaneous mutations in *E. coli* wild-type occur at the average rate of 0.0022 mutation per hour, suggesting that it takes almost 19 days

for 1 mutation to happen. Moreover, the μMA experiment allowed them to overcome the bias associated with traditional MA studies, where natural selection eliminates all lethal mutations, demonstrating that the majority of accumulated mutations were non-deleterious to the cell, while only 1% were lethal (Robert *et al.*, 2018). Moreover, visualization and analysis of replication errors also revealed that the latter emerge in rapidly growing bacteria heterogeneously, with majority occurring in subpopulations of cells confronting endogenous stresses (Woo *et al.*, 2018).

Finally, the introduction of maximum-depth sequencing (MDS) allowed direct high-throughput sequencing of regions of interest, enabling detection of rare variants in population of cells and evaluation of variance in the rate of mutations across genome. Such analysis of multiple loci revealed that mutation rates vary by almost ten times across the *E. coli* genome (Jee *et al.*, 2016). Thus, emerging technologies will allow us to get a deeper insight into the nature of mutations, as well as their interactions and effects on fitness of an organism. This will undoubtedly benefit us in understanding the process of evolution already in the nearest future.

1.2.2 Origins of mutations

1.2.2.1 Replication errors

Spontaneous mutations occur at a rate 10^{-10}–10^{-9} per base per cell per generation in many bacteria, demonstrating the extreme fidelity of the replication process that occurs in living cells *in vivo* (Drake *et al.*, 1991; Schroeder *et al.*, 2017). Spontaneous mutations mainly result from the errors that arise during DNA replication (Kunkel, 2009). Since replication errors are generated by DNA polymerases, one might say that polymerases shape the landscape of the genome and introduce variability that drives adaptation and evolution. However, the accuracy of replication also depends on the integrity of DNA itself, since DNA polymerases use DNA strand as a template to select dNTPs for incorporation during replication and repair processes. In addition to replication, DNA is also a template for other processes, including transcription, recombination and repair, but it is also constantly challenged by various DNA damaging events (Lindahl and Nyberg, 1972; Lindahl, 1993; Aguilera, 2002; Maki, 2002; Preston *et al.*, 2010; Aguilera and Gaillard, 2014). Hence, complex transactions between these processes determine integrity of the molecule, while any perturbations can have dramatic consequences on the replication fidelity and overall stability of the genome. Nevertheless, the major mechanism that assures the accuracy of DNA replication is the intrinsic fidelity of DNA polymerases involved in this process (Kunkel, 2009). DNA duplication is mediated by replicative high-fidelity DNA polymerases, supreme fidelity of which is achieved by (i) the selection and incorporation of the correct nucleotide, which is a primary determinant of fidelity; and (ii) exonuclease proofreading activity that mediates the excision of

any incorrectly inserted nucleotides, as was thoroughly discussed in Chapter 1.1.1.1 of this thesis. Following DNA synthesis, the fidelity of replication is further ensured by post-replicative DNA mismatch repair (MMR), which corrects polymerase errors (misincorporations, pre-mutations) that escaped proofreading (Fig. 4) (Kunkel and Erie, 2005; Fijalkowska *et al.*, 2012). For example, in *Bacillus subtilis* (*B. subtilis*) MMR occurs at the replisomes, demonstrating that in some organisms DNA repair might be directly coupled to replication (Liao *et al.*, 2015). In addition to MMR, proofreading proficient polymerases may contribute to overall replication fidelity by mediating repair of errors introduced by other polymerases during the process of replication, and thus play essential role in maintaining genome stability (Tago *et al.*, 2005; Fujii and Fuchs, 2007; Reha-Krantz, 2010). Initial nucleotide selectivity accounts for up to 10^{-4}–10^{-6} error rate, exonucleolytic proofreading activity to 10^{-2} and postreplicative correction by MMR to about 10^{-2}–10^{-3}. Together these events yield a phenomenally low overall error rate of 10^{-9}–10^{-11} per bp during high-fidelity DNA replication (Fig. 4) (Kunkel and Bebenek, 2000; Kunkel, 2004; Herr *et al.*, 2011; Fijalkowska *et al.*, 2012).

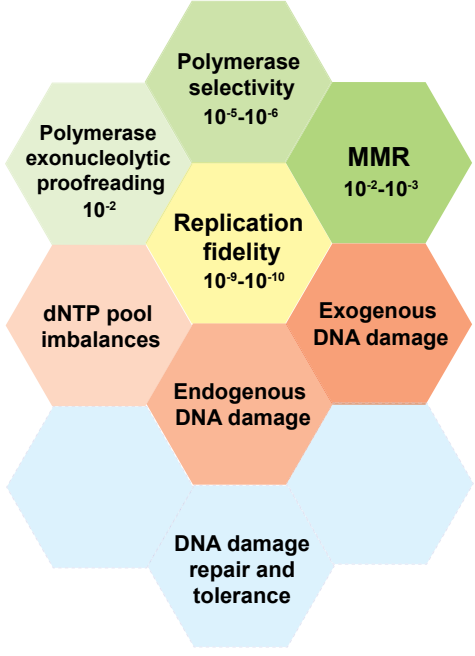

Figure 4. Main determinants of the overall DNA replication fidelity. Interplay between mechanisms that promote high fidelity replication (indicated in green) and prevent genome instability (DNA damage repair) that can result from various factors indicated in red, determine the overall replication fidelity.

1.2.2.2 Nucleotide pool as a precursor of mutations

The accuracy of DNA synthesis highly relies on the optimal dNTP pool in the cell (Schaaper and Mathews, 2013; Pai and Kearsey, 2017). Since polymerase nucleotide selectivity is an important mechanism of intrinsic fidelity, any alterations in nucleotide pool concentrations can affect the correct-incorrect dNTP ratio and result in increased mutagenesis (Wheeler *et al.*, 2005; Gon *et al.*, 2011; Schaaper and Mathews, 2013). Not only imbalances in the particular dNTP concentration (Miller *et al.*, 2002), but also increased concentration of all dNTPs can provoke rise in spontaneous mutation rates (Gon *et al.*, 2011). Elevated dNTP concentrations can facilitate the ability of polymerase to extend mismatched primer termini and allow to incorporate the nucleotide across DNA damage, *i.e.,* mediate damage bypass (Mertz *et al.*, 2015; Nevin *et al.*, 2017). As such, the ribonucleotide reductases, which control the synthesis and maintain the concentration of dNTPs in the cell, are one of the determinants of DNA fidelity (Mathews, 2014). A second critical aspect concerning dNTP pool is the fact that free dNTPs are more easily damaged than DNA by toxic by-products of normal cellular metabolism, especially by oxidative damage (Haghdoost *et al.*, 2006). To avoid incorporation of modified mutagenic dNTPs into DNA during replication and minimize spontaneous mutagenesis, "house-cleaning" enzymes act as a sanitizers of the nucleotide pool (Michaels *et al.*, 1992; Galperin *et al.*, 2006). In general, replicative DNA polymerases have low efficiency in incorporation of oxidized nucleotides into DNA, however *E. coli* replicative Pol III, member of C-family polymerases, is an exception, effectively misincorporating oxidized guanine opposite adenine during DNA replication (Katafuchi and Nohmi, 2010; Yamada *et al.*, 2012; Markkanen, 2017). Moreover, low-fidelity Y-family TLS DNA polymerases seem to favour and efficiently incorporate oxidized dNTPs opposite DNA template, and, for example, the use of oxidized nucleotides by *E. coli* Pol IV under certain conditions can be not only mutagenic, but also lethal to cells (Yamada *et al.*, 2006; Katafuchi and Nohmi, 2010; Foti *et al.*, 2012).

Despite high accuracy of replicative polymerases, some base pair mismatches are still incorporated during the DNA synthesis. The presence of rare tautomeric analogues of normal DNA bases (energetically unfavored tautomeric and anionic forms) in cells can lead to the deviations from normal W-C pairing (Kimsey *et al.*, 2015). For example, dT-dGTP or dA–dCTP W-C-like mispairs mimic the W-C geometry of the cognate base pair in the insertion state during the incorporation step (W. Wang *et al.*, 2011; Kimsey *et al.*, 2015). Such mismatches can be efficiently incorporated by high-fidelity DNA polymerases during DNA synthesis, supporting the occurrence of spontaneous base substitutions (Bebenek *et al.*, 2011). In addition, the genomic integrity strongly depends on the ability of polymerase to select against rNTPs to prevent their incorporation during DNA synthesis (Joyce, 1997). However, even under normal conditions, replicative DNA polymerases-mediated rNTP incorporation is the most common replication error (Nick McElhinny *et al.*, 2010). The

presence of ribonucleotides in the genome can alter genomic information, interfere with normal DNA replication, transcription and repair processes, and also diminish the backbone stability (Li and Breaker, 1999; Potenski and Klein, 2014). For example, *E. coli* Y-family polymerase Pol V has low sugar selectivity and misincorporates rNTPs with high frequency, and in order to protect genome integrity, cells have acquired different mechanisms to decrease the mutagenic effect of redundant rNTPs (McDonald *et al.*, 2012).

1.2.2.3 DNA damage as a precursor of mutations

Although DNA is the cradle of genetic information, molecule itself is highly unstable due to its nature (Lindahl, 2016). For example, about 10^4–10^5 lesions are produced in a single mammalian cell every day (Lindahl and Barnes, 2000; Preston *et al.*, 2010). These DNA lesions mostly result from spontaneous hydrolysis of DNA bases and endogenous damage formed during the normal cellular metabolism (Lindahl, 1993; Friedberg, 2008). The third source of DNA damage is exogenous in origin, as various external stressors, like UV light and plethora of chemicals and agents, can damage DNA either directly or indirectly (Lindahl, 1993; Friedberg, 2008).

Hydrolytic deamination of DNA bases is an important spontaneous reaction, leading to mutagenesis. Cytosine hydrolytically converts to uracil, guanine to xanthine and adenine to inosine (Lindahl, 1993). Mutagenic deamination products of cytosine and adenine can pair with adenine and cytosine respectively and lead to transition mutations (Duncan and Miller, 1980; Iyama and Wilson, 2013). Moreover, such spontaneous deamination of cytosines to uracils occurs in ssDNA more than 100 times frequently than in double-stranded DNA (dsDNA) (Frederico *et al.*, 1990; Beletskii and Bhagwat, 1996). Spontaneous depurination (loss of purine bases in DNA) leads to the appearance of cytotoxic and potentially mutagenic apurinic/apyrimidinic (AP) sites at the rate of almost 10^4 per mammalian cell per single day (Lindahl and Nyberg, 1972; Hevroni and Livneh, 1988). In addition to spontaneous hydrolysis and deamination, endogenously produced reactive oxygen species (ROS) and non-enzymatic alkylation are the main contributors to spontaneous DNA damage (van Loon *et al.*, 2010; Tubbs and Nussenzweig, 2017). ROS introduces a high variety of DNA lesions, including base modifications and DNA breaks, accounting for a significant portion of spontaneous mutations in aerobically growing *E. coli* cells across the whole genome (Sakai *et al.*, 2006; van Loon *et al.*, 2010; Lee *et al.*, 2012; Foster *et al.*, 2015). The most abundant ROS-induced lesion, 8-hydroxy-2'-deoxyguanosine (8-OH-dG), is extremely mutagenic, since during DNA synthesis replicative polymerases highly efficiently (mis)incorporate adenine across 8-OH-dG in the DNA template. Such mispair mimics the normal base pair and is not recognized by exonucleolytic activity of polymerase, leading to the appearance of G:C→T:A mutations (Shibutani *et al.*, 1991; Maki and Sekiguchi, 1992; Hsu *et al.*, 2004).

Endogenously formed alkylating agents such S-Adenosylmethionine (SAM), an important methyl donor for enzymatic reactions, or those generated as a result of lipid peroxidation or nitrosation reactions, represent an important unavoidable source of DNA damage (Barrows and Magee, 1982; Taverna and Sedgwick, 1996). Alkylating agents transfer the alkyl group on different cellular molecules, including DNA, forming adducts on nitrogen and oxygen atoms of DNA bases, which might be toxic and mutagenic to cell (Drabløs *et al.*, 2004). The most frequent *N*-methylation product, N^7-methylguanine (N^7meG), is relatively harmless itself, however, adduct destabilizes the *N*-glycosidic bond, making it more susceptible to hydrolysis that can lead to the formation of AP sites or imidazole ring-opened derivative of guanine and inhibition of DNA synthesis (Tudek *et al.*, 1992; Lindahl, 1993; Tudek, 2003). Another important lesion, N^3-methyladenine (N^3meA), is highly toxic to cells, forming a replication block to DNA and RNA polymerases (Wyatt and Pittman, 2006). *O*-alkylation adducts are highly mutagenic, and the most common O^6-methylguanine (O^6meG) and O^4-methylthymine (O^4meT) lesions lead to G:C→A:T and T:A→C:G mutations, respectively (Marnett and Burcham, 1993; Lindahl, 1996).

All spontaneous and damage-induced toxic and (pro)mutagenic alterations in DNA must be repaired a priori to DNA replication to maintain genomic integrity (Preston *et al.*, 2010). To tackle that, cells are well equipped with various DNA repair mechanisms (Friedberg *et al.*, 2006). Still, some damage left unrepaired, represents challenges for high-fidelity replicative polymerases and can lead to perturbations in DNA replication, and be lethal to cells. Unrepaired DNA lesions can transiently be converted into (pre)mutations by specialized DNA polymerases present in cells via translesion DNA synthesis, and subsequently fixed into mutations during the next round of replication (Maki, 2002; Pagès and Fuchs, 2002). Damage can also trigger up-regulation of DNA damage response, which represents complex network of highly accurate DNA repair pathways and potentially mutagenic damage tolerance processes (Janion, 2008; Kreuzer, 2013; Hanawalt, 2015). In response to DNA damage cells also elevate dNTP pool size, and such increase affects the fidelity DNA replication, as was described above (Gon *et al.*, 2011). Thus, the maintenance of DNA integrity is not only contingent on the accuracy of DNA polymerases, but likewise on the proper and timely repair of any occurring DNA damage.

1.2.3 Insights into mutational topology of DNA

Studies of mutational data across the entire genome of various non-mutator bacteria have revealed that the most frequent spontaneous mutation type are base substitutions, in particular, G:C→ A:T transitions (Lee *et al.*, 2012; Long *et al.*, 2015; Sung *et al.*, 2015). Because part of the spontaneous mutations is eliminated by MMR, the analysis of MMR-defective strains allows revealing the true errors made by polymerases during the process of DNA replication. The spectrum of mutations in MMR-deficient strains is shifted from G:C→ A:T

transitions observed in wild-types to A:T→ G:C transitions, indicating that MMR prevents drifting of genomes to higher G:C content (Lee *et al.*, 2012; Long *et al.*, 2014; Sung *et al.*, 2015). Interestingly, in naturally devoid of MMR *Mycobacterium smegmatis*, the mutation rate is similar to those observed in other bacteria, suggesting the contribution of other mechanisms to genomic integrity maintenance. On the other hand, the spectrum is shifted towards A:T→ G:C transitions (Kucukyildirim *et al.*, 2016), similarly to those observed in MMR-deficient strains (Lee *et al.*, 2012; Long *et al.*, 2014, 2015; Sung *et al.*, 2015).

Genome analysis of MMR-deficient *E. coli* (Foster *et al.*, 2013)*, P. aeruginosa* (Dettman *et al.*, 2016) and *Pseudomonas fluorescens* (Long *et al.*, 2014) also demonstrated non-random distribution of mutations across the genome. These studies revealed symmetrical pattern of bi-modal distribution of mutations: the lowest mutation rates were found next to the origin of replication, while mutational peaks were present at adjacent to terminal and intermediate regions. Such correlation in heterogeneity in the distribution of mutations across the genome suggests the existence of shared mechanism of replication bias.

1.2.3.1 Sequence context-dependent mutation bias

Local sequence composition has a strong impact on the fidelity of DNA polymerases (Sung *et al.*, 2015; Schroeder *et al.*, 2016). Recent whole-genome analysis of MA lines demonstrate that the upstream and downstream nucleotides of the "triplet" sequence context influence the fidelity of nucleotide selection, and thus affect local spontaneous mutation rate (Sung *et al.*, 2015; Merrikh *et al.*, 2016; Schroeder *et al.*, 2016). Some of the "triplet" sequences accumulate mutations at higher rate, demonstrating the existence of context-dependent mutation patterns that might be one of the main determinants of mutagenesis (Schroeder *et al.*, 2016). For example, whole-genome analysis of MA lines in *B. subtilis* and *P. aeruginosa* have revealed that sites, adjacent to G:C base pairs, have significantly elevated mutation rates (Sung *et al.*, 2015; Dettman *et al.*, 2016). GATC sites (canonical motifs involved in methylation) can also influence mutation rates, serving as hotspots for A:T transversions (Lee *et al.*, 2012). Sequence context also significantly influences the mutagenicity of the major oxidative lesion, 8-OH-dG (Foster *et al.*, 2015). One of the explanations of such bias can be attributed to the stability of DNA base pairings, known as base stacking, with A:T pairs being more destabilizing of DNA duplex than G:C pairs in the presence of a mismatch (Yakovchuk *et al.*, 2006). Therefore, G:C base pair-flanking mismatches can be skipped due to the reduced helix disturbance and the ability to be detected and proofread by the polymerase, suggesting that base pairings and base-stacking can have an important role in replication errors and spontaneous mutagenesis (Dettman *et al.*, 2016).

1.2.3.2 Leading- and lagging-strand replication bias

As a consequence of bi-directional process of replication, the leading- and lagging-strands have different mutational biases (Xia, 2012; Dettman *et al.*, 2016). For example, analysis of many circular bacterial genomes have revealed the existence of asymmetry in GC composition, with the excess of G over C (and T over A) in the leading-strand, with the reverse for the lagging-strand, known as "GC skew" (Lobry, 1996; Guo and Ning, 2011). Differences in replication modes, fidelity of the lagging- and leading-strand replication and repair efficiencies were suggested to explain these patterns (Fijalkowska *et al.*, 1998; Reijns *et al.*, 2015). For example, replication on the lagging-strand in *E. coli* is more accurate than the leading-strand replication (Gawel *et al.*, 2014; Maslowska *et al.*, 2018). Latter study suggests that such strand composition bias could be associated with the different frequency of the deamination of cytosines, which occurred predominantly in the lagging-strand template, leading to the appearance and accumulation of C to T mutation, subsequent cytosine loss resulting in GC skew (Bhagwat *et al.*, 2016).

Another important mechanism that can lead to the bias in the nucleotide composition may be linked to transcription, as replication-transcription conflicts may promote mutagenesis (Paul *et al.*, 2013; Million-Weaver *et al.*, 2015). Both replication and transcription share DNA as a template, and in actively dividing cells collision conflicts between DNA replication and transcription machineries represent an important source of DNA damage associated with genomic instability (García-Muse and Aguilera, 2016). Lagging-strand genes are transcribed in the opposite direction to DNA replication movement (head-on), resulting in frequent collisions of replication and transcription machineries. Due to the constant replication-transcription conflicts, these, so-called head-on genes, mutate and evolve at higher rates than those transcribed co-directionally (Paul *et al.*, 2013; Million-Weaver *et al.*, 2015). Because RNA transcription is 10–20 times slower than the movement of the replisome, the co-directional leading-strand replication-transcription conflicts can also occur, but with lower frequency and milder outcome (Soultanas, 2011; Merrikh, 2017). The head-on gene orientation may likely increase mutagenesis within a given gene or in the promoter region, demonstrating that replication-transcription conflicts can also modify gene expression patterns (Paul *et al.*, 2013; Million-Weaver *et al.*, 2015; Sankar *et al.*, 2016). One of the transcription-replication collision mechanisms underlying increased mutagenesis in lagging-strand genes represents the involvement of error-prone Y-family DNA polymerase in transcription-coupled nucleotide excision repair (TC-NER), as one study in *B. subtilis* suggests (Million-Weaver *et al.*, 2015). Interestingly, in bacteria, most of the essential and highly transcribed (*i.e.*, protein-encoding) genes are located in the leading-strand, demonstrating the co-directional nature of transcription and replication processes (Rocha, 2004, 2008; Merrikh, 2017). On the other hand, many genes located in the lagging-strand are highly induced only under stressful conditions. Therefore, it could be one of the global mechanisms, leading to the increase in

mutation rates of specific genes during stress, which are important for fitness and evolution (Merrikh, 2017). The phenomenon of this process will be discussed in Chapter 1.5 of this thesis.

Taken together, these surprising and interesting results from recent whole-genome studies expanded our knowledge of how new mutations shape the genome. In addition, they open new conceptual frameworks to study the causes of variation and to reveal to which extent this variation is introduced. Thus, future research will be beneficial for understanding biological significance of mutation in fitness and disease.

1.3. Translesion DNA synthesis

Translesion DNA synthesis (TLS) represents a mechanism, by which DNA lesion is bypassed by incorporation of a nucleotide opposite the lesion (Waters *et al.*, 2009). TLS represents one of the DNA damage tolerance mechanisms that allows cell to complete DNA replication in the presence of DNA damage (Fuchs and Fujii, 2013; Bi, 2015). TLS is mediated by specialized DNA polymerases, most of which belong to Y-family polymerases, the existence and function of which have been puzzling scientists for many years.

The existence of inducible "mutation-prone" DNA replication mechanism ("SOS repair" hypothesis) was first proposed by Miroslav Radman in privately circulating letter already in 1970 (Bridges, 2005). The hypothesis of mutagenic damaged DNA repair was further supported by the isolation and characterization of UV-nonmutable (*umu*$^{-}$) mutants in *E. coli* (Kato and Shinoura, 1977; Steinborn, 1978). In the beginning of the 1980-s it was shown that *umuC* and *umuD* are induced in cells upon DNA damage as a part of SOS response, required for DNA damage-induced mutagenesis (Sommer *et al.*, 1993), however mechanism behind that was not understood. Year 1999 was a turning point in this story, when *dinB*-encoded Pol IV in *E. coli* (Wagner *et al.*, 1999); Rad30 in *S. cerevisiae* (Johnson *et al.*, 1999) and *XP-V*-encoded pol η in human (Masutani, Araki, *et al.*, 1999; Masutani, Kusumoto, *et al.*, 1999) were shown to be *bona fide* "error-prone" DNA polymerases, displaying intrinsic lesion-bypassing powers. In addition, two independent studies revealed that UmuC was also a DNA polymerase activated by UmuD′, and demonstrated the ability of UmuD′$_2$C complex (named Pol V) to copy damaged and non-damaged DNA (Reuven *et al.*, 1999; Tang *et al.*, 1999). Shortly after, new members of this newly discovered UmuC/DinB/Rad30/Rev1 superfamily of DNA polymerases were identified through all domains of life, demonstrating the evolutionary conservation of translesion DNA synthesis mechanism, and since then they are referred to as "Y-family" of DNA polymerases (McDonald *et al.*, 2001; Ohmori *et al.*, 2001). In recent years our understanding of biological function of TLS polymerases has extended beyond their canonical translesion activities in DNA damage tolerance, which were historically considered to be mutagenic (Goodman and Woodgate, 2013). The unexpected roles of TLS polymerases have been discovered in DNA repair-associated processes, regulation of replication check-

points and replication of undamaged structured DNA in unstressed eukaryotic cells in both disease and fitness and in the context of genomic integrity maintenance (Yousefzadeh and Wood, 2013; Pillaire *et al.*, 2015; Wickramasinghe *et al.*, 2015). Despite the great progress in this field, we still do not have a complete idea on the complexity and amazing variability of their functions and transactions that ensure stability and duplication of the genomes.

1.3.1 General features of Y-family polymerases

Y-family DNA polymerases are phylogenetically divided into six groups (Fig. 5) (Yang, 2014). They are typified by (i) *E. coli* DinB (Pol IV), the most extensively distributed branch found in archaea, bacteria and eukaryotes, (ii) UmuC, which is present only in prokaryotes (the catalytic subunit of *E. coli* Pol V), and (iii) enzymes found only in eukaryotes, such as pol η (encoded by *POLH*), pol ι (*POLI*), pol κ (*POLK*) and Rev1 (Ohmori *et al.*, 2001; Sale *et al.*, 2012).

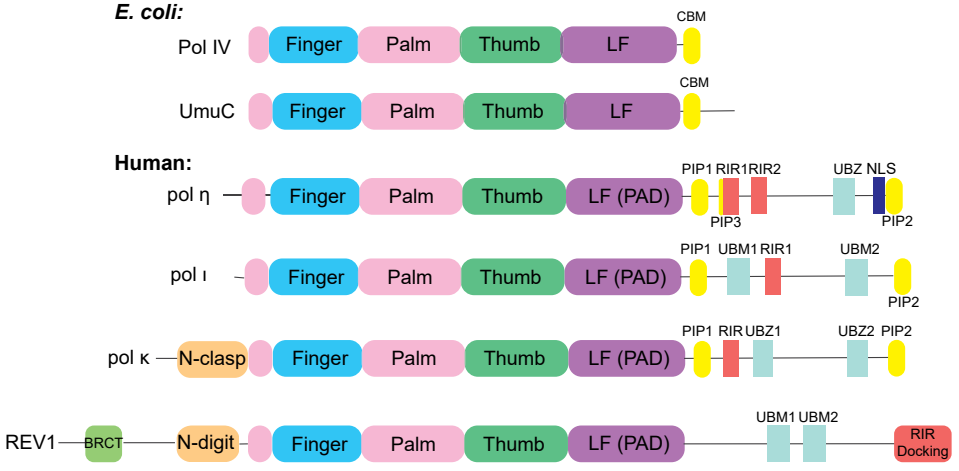

Figure 5. Domain structures of Y-family polymerases. Main domains in representatives of six subgroups of Y-family polymerases are visualized. Abbreviations: LF – little finger domain; PAD – polymerase-associated domains in eukaryotic polymerases. NLS in pol η is a nuclear localization signal; PIP motif stands for proliferating cell nuclear antigen (PCNA)-interacting peptide; UBZ/UMB are ubiquitin binding/ubiquitin-binding zinc finger motifs; RIR motif is a Rev1-interacting region. BRCT denotes BRCA1 C-terminal domain, involved in Rev1 interaction with PCNA, and RIR-docking in REV1 is a C-terminal domain involved in interaction with other TLS polymerases. Based on (Yang, 2014; Trakselis *et al.*, 2017).

The distinctive functions of Y-family DNA polymerases are associated with their unique structure. They share little sequence homology with high-fidelity replicases, but still retain similar conserved polymerase core “right hand” topology and a common catalytic mechanism (Fig. 1A and Fig. 6) (Ling *et al.*,

2001; Prakash *et al.*, 2005). In contrast to replicative DNA polymerases, Y-family polymerases contain two domains: the canonical N-terminal catalytic polymerase domain and the unique C-terminal domain that contributes to DNA binding and is critical for the full polymerase activity and fidelity (Fig. 5 and Fig. 6) (Ling *et al.*, 2001; Silvian *et al.*, 2001; Wilson *et al.*, 2013). The polymerase core, which includes catalytic region, is about 350–500 amino acid (aa) residues long and is conserved among family, while the C-terminal regulatory region varies in size from 10 to 600 aa residues and is less conserved across family members (Fig. 5) (Yang, 2014). C-terminal domain is designated as "little finger" (LF) in archaeal and bacterial enzymes, and in eukaryotic proteins it is known as polymerase-associated domain (PAD) (Ling *et al.*, 2001; Trincao *et al.*, 2001; Bunting *et al.*, 2003).

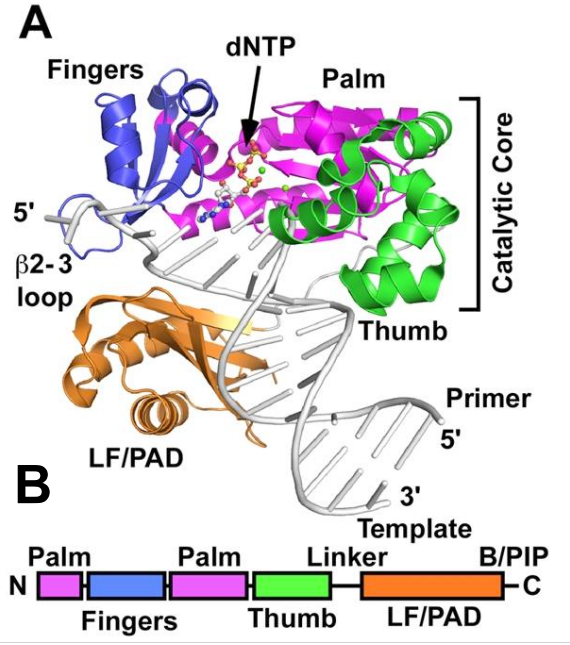

Figure 6. Structural overview of a model Y-family DNA polymerase, Dpo4. Ternary complex of Dpo4 in complex with DNA and incoming dNTP (A) and linear diagram of the structural domains of the polymerase (B). (A) N-terminal catalytic domain is comprised of the "palm" (magenta), "fingers" (blue), and "thumb" (green) subdomains, which is linked to the C-terminal LF/PAD domain (orange) by the linker peptide. The primer and template DNA are indicated in white, the incoming nucleotide is represented in ball and stick pattern. (B) The β-clamp/PCNA interaction (B/PIP) sequence is located at the C-terminus of the protein. Adapted by permission from (Pata, 2010).

At the C-terminal region preceded by LF/PAD subdomain, all Y-family polymerases, with the exception for Rev1, possess a short region needed for interaction with processivity β-clamp in bacteria (clamp-binding motif, CBM) or with proliferating cell nuclear antigen (PCNA) in eukaryotes and archaea (PIP, PCNA-interacting protein) (Fig. 5 and Fig. 6) (Dalrymple *et al.*, 2001; Pata, 2010). Both "thumb" and LF/PAD subdomains grip the primer-template DNA, where the "thumb" subdomain associates with the DNA across the minor groove, while the LF/PAD domain interacts with the major groove of DNA (Fig. 6A) (Pata, 2010).

Similarly to high-fidelity DNA polymerases, the amino acid residues that coordinate metal ions, required for the catalytic reaction, are located in the "palm" subdomain (Ling *et al.*, 2001). However, the "finger" and "thumb" subdomains of Y-family members are strikingly smaller and stubbier compared to high-fidelity polymerases (Ling *et al.*, 2001; Trincao *et al.*, 2001; McCulloch and Kunkel, 2008), resulting in very little or virtually no contact between DNA and incoming nucleotide, allowing dNTP and PPi to freely diffuse in and out (Ling *et al.*, 2001; Sale *et al.*, 2012; Yang and Gao, 2018). Due to the smaller size of the "finger" subdomain, the active site of Y-family polymerases differs remarkably from high-fidelity polymerases: it is much wider and more open, allowing to accommodate aberrant DNA structures (Silvian *et al.*, 2001). The active site is also relatively rigid and already preformed (Ling *et al.*, 2001; Trincao *et al.*, 2001; Yang, 2005). Moreover, crystallographic structural studies demonstrated that the "finger" subdomain almost does not undergo the conformational change from an open to a closed state as seen in other DNA polymerase families upon nucleotide binding, suggesting the absence of conformational selection mechanism of the incoming nucleotide (Yang, 2003; Rechkoblit *et al.*, 2006; Wong *et al.*, 2008; Wilson *et al.*, 2013). In some of the Y-family members, *e.g.,* archaeal *Sulfolobus solfataricus* DNA polymerase IV (Dpo4) and human pol κ, representatives of the DinB branch of Y-family polymerases, nucleotide binding and incorporation induces the rotation of the LF/PAD relative to the polymerase core (Lone *et al.*, 2007; Wong *et al.*, 2008; Chu *et al.*, 2014). LF/PAD subdomain is connected to the catalytic polymerase domain by a highly flexible peptide linker (Fig. 6B). This linker designates the conformational specificity of the polymerase, and it might determine the enzymatic and biological function of the individual Y-family members (Wilson *et al.*, 2013). For example, change of only three amino acid residues of the inter-domain linker of *Sulfolobus* Y-family polymerase Dbh allowed to adopt the conformation of Dpo4 and affected the fidelity of the polymerase (Mukherjee *et al.*, 2014). This way, the active site of Y-family polymerase may be adapted to a wide range of DNA lesions by virtue of inter-domain linker flexibility, allowing binding of distorted DNA lesions (Wilson *et al.*, 2013).

The performed active site, loose fit of substrates and the lack of substrate-induced conformational checkpoint can explain the limited ability of Y-family polymerases to discriminate between the right and the wrong nucleotide in the active site, leading to low fidelity (Khare and Eckert, 2002; Wong *et al.*, 2008;

Yang, 2014). In addition, Y-family polymerases lack intrinsic 3′–5′ exonuclease proofreading activity (Goodman, 2002; Kunkel, 2009). Moreover, they all exhibit relatively poor processivity and low catalytic efficiency, especially when replicating undamaged DNA templates (Jarosz *et al.*, 2007). For example, the fidelity of an archaeal model Y-family polymerase Dpo4 is 8×10^{-4} to 3×10^{-4}, and it extends primer by only 1–2 nucleotides per binding event (Boudsocq *et al.*, 2001). On the other hand, these biochemical characteristics play an important role in maintaining the overall genome integrity by preventing error-prone DNA polymerases from replicating long stretches of DNA (Vaisman and Woodgate, 2017).

Despite the similarities in basic structure among Y-family polymerases, they differ in many other ways. Due to their specific structural characteristics, Y-family polymerases are also highly divergent in functional specificity (Prakash *et al.*, 2005). Different members of the Y-family polymerases possess different template-substrate specificities and translesion catalysis strategies, and the cognate DNA lesion might be bypassed with extremely high efficiency and accuracy (Jarosz *et al.*, 2006; Livneh *et al.*, 2010; Maxwell and Suo, 2014). At some point, enzymes can be partly typified by the insertional patterns opposite specific DNA lesions. For example, human pol η and *E. coli* Pol V pair, and pol κ and *E. coli* Pol IV pair are functional orthologues based on their similarity, efficiency and fidelity of dNTP insertion opposite specific types of DNA damage (Lee *et al.*, 2006; Chandani *et al.*, 2010; Sholder *et al.*, 2015). As such, accuracy of the lesion bypass will depend on the combination of a lesion and polymerase(s) involved in this process (Becherel and Fuchs, 2001; Ohmori *et al.*, 2009). Still, a lot of critical questions, regarding structure, substrate specificity and nucleotide selectivity of the variety of different Y-family polymerases, remain unanswered.

1.3.2 Mechanism and models of translesion DNA synthesis

The basic mechanism of TLS can be divided into three steps (Fig. 7A). First, the damaged nucleotide must be accommodated in the polymerase active site (pre-insertion complex). If damage is adapted, the next step involves selection and (mis)incorporation of dNTP opposite the DNA lesion (insertion step), followed by elongation/extension of the 5′ base beyond the insertion for one or several bases (extension step) (Woodgate, 2001; Sale *et al.*, 2012; Yang and Gao, 2018). All these steps are exclusively challenging in their ways: in the insertion step, distorted DNA lesion must be correctly adapted and accommodated for nycleotidyl reaction to happen. The elongation step is also tricky, since distorted lesion-dNTP base pair must be accommodated for subsequent primer extension (Becherel and Fuchs, 2001; Yang and Gao, 2018). Because of the complexity of DNA adducts the incorporation and extension steps can be performed by different TLS polymerases. Due to the unique structural features, Y-family polymerases are mostly involved in the insertion step; and the extension of the

primer termini with distorted base pair is performed by the members of B-family polymerases, such as Pol II in *E. coli* or pol ζ in eukaryotic cells (Shachar *et al.*, 2009; Wang and Yang, 2009; Livneh *et al.*, 2010). Rev1 has a unique non-catalytic accessory role, providing a docking site for other TLS polymerases and proteins to the replication fork, and facilitates polymerase exchange (Waters *et al.*, 2009). The most recently discovered PrimPol bypasses a wide range of DNA lesions either in canonical direct translesion way across the lesion or by virtue of a primase activity by re-annealing and re-initiation of DNA replication downstream of the DNA lesion (lesion skipping) (Boldinova *et al.*, 2017; Guilliam and Doherty, 2017). Therefore, the real picture of TLS, especially in mammalian cells, possessing multiple polymerases that are able to perform incorporation of dNTP opposite the lesion and/or extension of distorted primer termini, can be very complex (Livneh *et al.*, 2010).

Two different translesion DNA synthesis models were shown to exist in both mammalian and bacterial cells (Fig. 7B, C) (Friedberg *et al.*, 2005; Lehmann and Fuchs, 2006; Lehmann *et al.*, 2007; Quinet *et al.*, 2014). In some instances, TLS occurs directly at the stalled replication forks at DNA lesions and involves polymerase switching (replicative TLS or "polymerase switching" model; Fig. 7B). In the polymerase switching model, TLS polymerase replaces the stalled replicative DNA polymerase and mediates lesion bypass. When DNA damage is bypassed, the replicative DNA polymerase gets back on track, and replication continues (Lehmann and Fuchs, 2006; Waters *et al.*, 2009; Sale *et al.*, 2012). Therefore, TLS polymerases, acting at the stalled replication fork, rescue the latter from the collapse, preventing the formation of toxic DSB (Berti and Vindigni, 2016). Alternatively, in the "gap-filling model" (post-replicative TLS) (Fig. 7C), TLS polymerases are involved in the repair of lesion-containing ssDNA gaps outside the replisome context (Waters *et al.*, 2009; Fuchs, 2016). In this case, specialized polymerase is recruited to the β-clamp located at the primer terminus next to the lesion site (Fig. 7C). Binding to the β-clamp enables TLS polymerase to synthesise short DNA patch across and beyond the DNA lesion that can be further elongated by replicative polymerase to fill in the gap completely (Naiman *et al.*, 2016). Following the DNA damage bypass, lesion is removed by post-replicative DNA damage repair mechanisms (Waters *et al.*, 2009; Fuchs and Fujii, 2013). Therefore, in the second model, TLS polymerases seal the damage-containing gaps to ensure complete genome replication and cell division (Kreuzer, 2005; Lopes *et al.*, 2006).

To accomplish TLS, specialized polymerases must be bound to β-clamp to achieve enough processivity to be able to synthesize DNA patch (so-called TLS patch) of sufficient length during a single binding event (Becherel *et al.*, 2002; Fujii and Fuchs, 2004). Such TLS patch must be long enough to avoid excision by the proofreading activity of the replicative DNA polymerase (like *E. coli* Pol III) that upon re-binding senses the lesion-induced distortion within 4–5 nucleotides of the primer terminus. Otherwise, replicative polymerase-mediated TLS patch degradation will lead to the abortive lesion bypass trials (Becherel *et al.*, 2002; Fujii and Fuchs, 2004, 2007).

However, how is the choice of the specific pathway made? It has been proposed that polymerase-switching might take place during active DNA replication (Waters *et al.*, 2009). However, the latest *in vivo* studies using single-molecule fluorescence microscopy suggest that at least in *E. coli* TLS polymerases Pol IV and Pol V predominantly act at sites away from replisome, suggesting the involvement of TLS in post-replicative gap-filling (Robinson *et al.*, 2015; Henrikus *et al.*, 2018). These latest observations in living cells bring new questions and challenge many TLS regulation models that have been previously proposed based on *in vitro* studies.

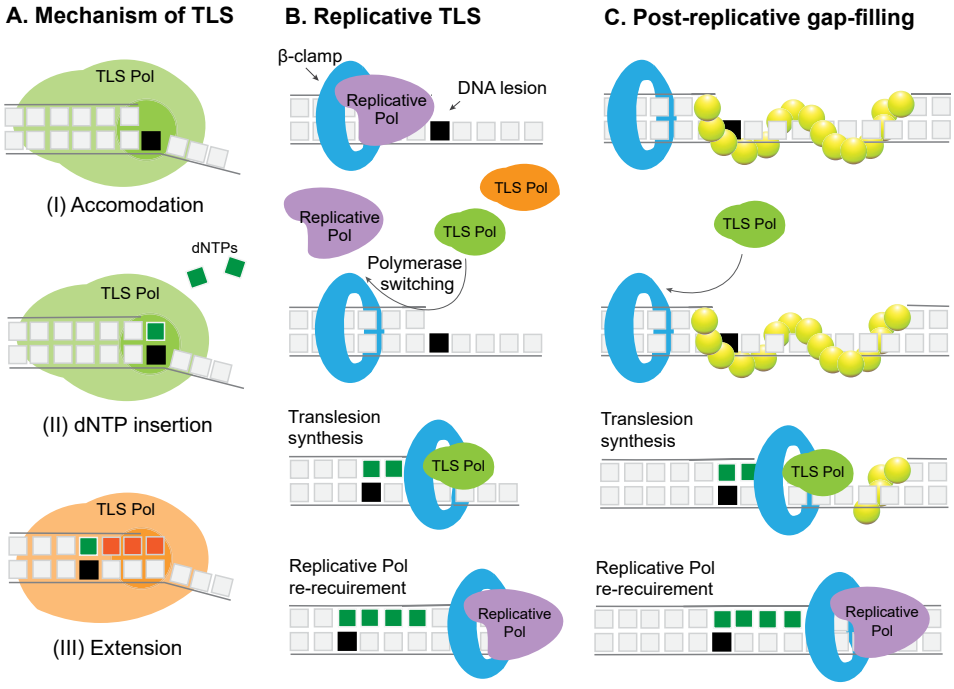

Figure 7. Mechanism of translesion DNA synthesis (TLS) (A) and two models for translesion DNA synthesis: replicative polymerase switching (replicative TLS) model (B), and post-replicative gap-filling model (C). Damaged nucleotide is designated as a black box; blue is a β-clamp, replicative polymerase indicated in purple and TLS polymerases in green and orange; SSB are indicated in yellow.

1.3.3 Regulation of TLS in *E. coli*

If the fidelity of TLS polymerase to replicate past specific DNA lesion can be very high, then replication of undamaged DNA is usually error-prone due to the lack of proofreading activity and unique kinetic and structural aspects of Y-family polymerases, discussed previously (Friedberg *et al.*, 2002). In addition, because of the non-coding or miscoding properties of most DNA lesions, TLS is inherently error-prone. Thus, the ability to survive in the presence of DNA

damage comes at the cost of increased mutation rate, and the access of TLS polymerases to DNA must be securely controlled by the cell to avoid extensive mutagenesis (Lehmann, 2006). The simplest control mechanism that mediates the action of TLS polymerases in cells is regulation of their concentration or availability, which in bacteria is regulated at different transcriptional and post-translational levels (Sale *et al.*, 2012; Goodman and Woodgate, 2013). As TLS represents DNA damage tolerance pathway, it is not surprising that stress response processes regulate and control amounts of TLS polymerases in cells. In the next chapter, the regulation of the best-studied SOS response-inducible TLS model in *E. coli* will be discussed.

1.3.3.1 SOS response regulation of TLS

Many bacteria, including *E. coli*, respond to various endogenous and exogenous stresses, which can damage DNA or interfere with DNA replication, with an inducible DNA damage response pathway termed SOS response (Sassanfar and Roberts, 1990; Erill *et al.*, 2007). The induction of the SOS response leads to coordinated up-regulation of genes involved in DNA damage repair, DNA damage tolerance pathways, and inhibition of replication and cellular division (Courcelle *et al.*, 2001; Crowley and Courcelle, 2002). As briefly was mentioned above, *E. coli* has five DNA polymerases, and three of them, Pol II, Pol IV and Pol V, are activated following induction of the SOS response (Goodman, 2002). The primary trigger of the SOS response is the accumulation of ssDNA (Baharoglu and Mazel, 2014). SOS response is regulated by the interplay of two proteins: LexA and RecA (Little and Mount, 1982). LexA is a transcriptional repressor, which in non-stressed cells interacts with its cognate LexA consensus sequences in the operator regions of LexA target genes, suppressing their expression (Brent and Ptashne, 1981; Walker, 1984). RecA is a recombinase that is responsible for homologous recombination and for mediating the derepression of LexA-controlled SOS genes (Lusetti and Cox, 2002; Cox, 2007). Upon DNA damage (or other processes that lead to ssDNA formation), RecA binds ATP and polymerizes the regions of ssDNA formed at the sites of DNA damage, and becomes conformationally activated (Fig. 8). RecA-ssDNA-ATP, or RecA* filament, serves as a co-protease and promotes self-cleavage of DNA-unbound LexA and its inactivation (Little *et al.*, 1981; Giese *et al.*, 2008; Gruenig *et al.*, 2008). Dissociation and cleavage of LexA from DNA initiates derepression of genes and activation of the SOS response (Butala *et al.*, 2011).

The SOS response is modulated through differential temporal activation of genes (Friedman *et al.*, 2005). Such differential induction depends on different binding affinity of the repressor LexA to its binding sites, which may vary in sequence and in the number of LexA boxes present at the promoter region of the gene (Ronen *et al.*, 2002; Kamenšek *et al.*, 2010). In this way, genes with low-affinity of LexA binding are expressed first, while the induction of genes with high LexA binding affinity occurs later.

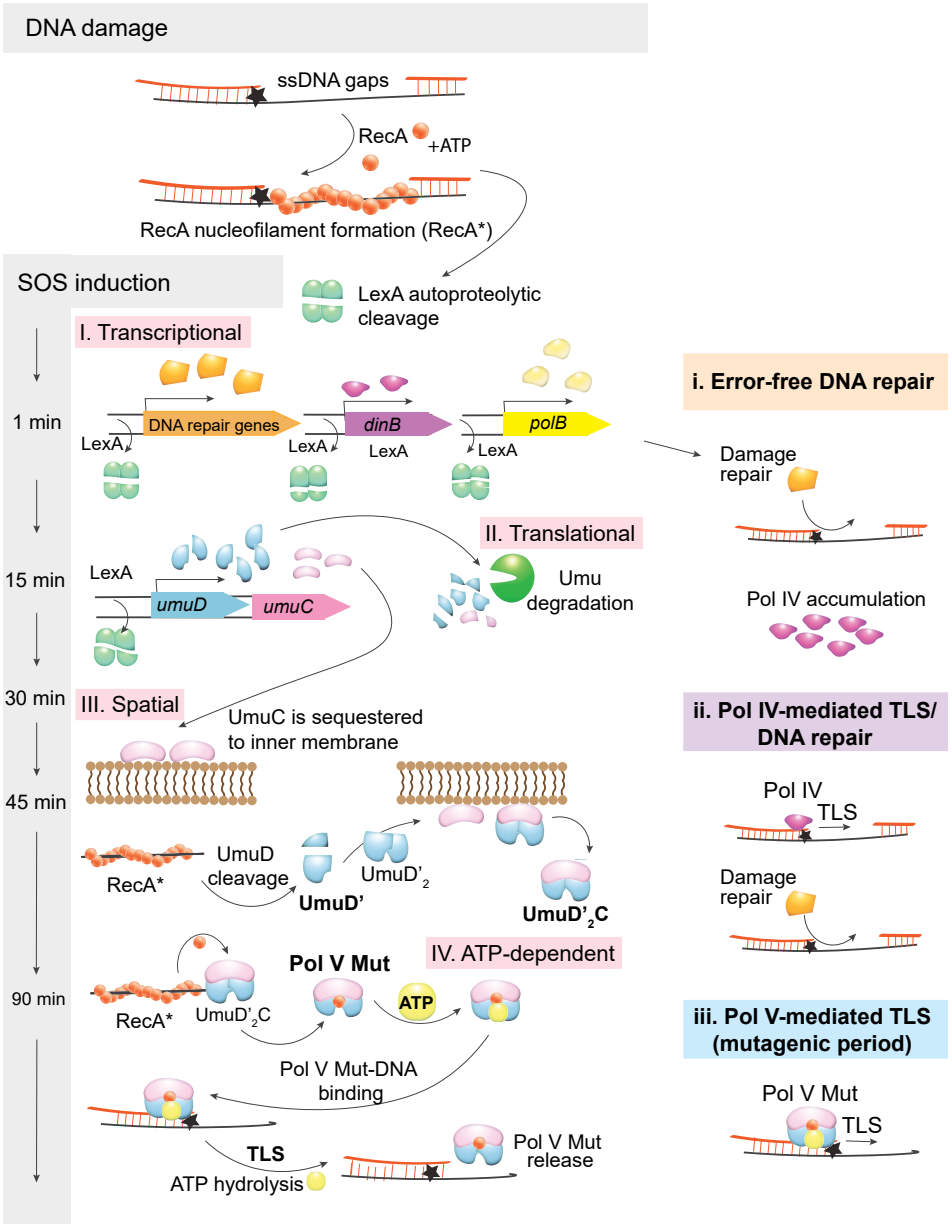

Figure 8. SOS response model and regulation of Pol V in *E. coli* cells. Figure is based on the studies of (Goodman, 2014; Robinson *et al.*, 2015; Goodman *et al.*, 2016; Henrikus *et al.*, 2018).

There is an interesting pattern in the chronology of the regulon activation: genes responsible for an error-free DNA repair, such as NER, are induced already within the first minutes after DNA damage, promoting survival in a high-fidelity manner; however, potentially mutagenic damage tolerance pathways are

induced later (Janion, 2008). Intriguingly, *E. coli polB*-encoded Pol II and *dinB*-encoded Pol IV, are also induced among the first ones (Fig. 8) (Fernández De Henestrosa *et al.*, 2000). The early expression in the SOS response and relatively high abundance of Pol IV and Pol II in unstressed cells suggests their possible involvement in TLS past endogenous DNA damage that can be generated during normal cell metabolism (Kim *et al.*, 2001; Goodman and Woodgate, 2013). In contrast to Pol II and Pol IV, *umuDC* operon, encoding mutagenic Pol V (UmuD′$_2$C) is induced late in the SOS response as a backup mechanism, when the cellular ability to repair DNA damage has been surpassed (Fig. 8) (Courcelle *et al.*, 2001, 2005; Robinson *et al.*, 2015). Pol V can replicate past various chemical- and UV-induced lesions, but with quite low fidelity (Tang *et al.*, 2000). Consequently, Pol V is responsible for the most of the damage-induced mutagenesis in *E. coli* that accompanies the SOS response (Kato and Shinoura, 1977). In addition, it is much more mutagenic on undamaged templates, being responsible also for the untargeted mutagenesis under SOS conditions (Maor-Shoshani *et al.*, 2000). Thus, to limit the mutagenic activity of Pol V and to keep expression to minimum in undamaged cells, activation of Pol V is tightly regulated at numerous complex levels (Goodman *et al.*, 2016; Jaszczur *et al.*, 2016). The transcriptional activation of the *umuDC* operon occurs only after 15 min after SOS induction, whereas catalytically active Pol V accumulates significantly later (Fig. 8) (Sato *et al.*, 1985; Sommer *et al.*, 1998). Such delay in the accumulation of active Pol V in cells is regulated on posttranslational level: both UmuC and UmuD′ proteins are constantly being degraded inside the cell by proteases (Frank *et al.*, 1996). Only 30–45 min after SOS induction, fraction of UmuC protein starts to appear, however, it is sequestered transiently at the inner cell membrane, which results in a further delay of Pol V activation (known as spatial regulation; Fig. 8) (Robinson *et al.*, 2015). Subsequently, RecA* filament-mediated cleavage of UmuD to its mutagenic UmuD′ form occurs. Formed UmuD′$_2$ associates with UmuC, leading to the release of the protein from membrane to cytosol and formation of resistant to proteolytic degradation UmuD′$_2$C complex, or Pol V (Fig. 8) (Robinson *et al.*, 2015). To achieve the final conversion, a single RecA subunit is transferred from the 3′– proximal tip of RecA* filament to Pol V, forming an active mutasome, composed of UmuD′$_2$C-RecA-ATP, referred to as Pol V Mut (Jiang *et al.*, 2009), which start to appear approximately 90 min after DNA damage (Robinson *et al.*, 2015). Pol V Mut was first found to be a DNA-dependent ATPase, which requires ATP to bind to DNA template (Fig. 8). It is the only polymerase described so far that possesses such intrinsic ATPase activity and autoregulatory mechanism (Erdem *et al.*, 2014). Such internally regulated DNA-dependent hydrolysis of ATP also restricts mutagenic activity of Pol V. Internal ATP hydrolysis releases the Pol V Mut complex from DNA, ensuring limited and restricted active polymerase time on the template (Erdem *et al.*, 2014). A fresh RecA* can reactivate deactivated Pol V Mut, if needed, until the SOS is switched off and the levels of RecA* are diminished (Gruber *et al.*, 2015). Such complex regulation allows cells first to deal with DNA damage using non-

mutagenic DNA pathways before error-prone DNA damage tolerance strategies in the face of mutagenic Pol V come into play.

One recent study has emerged with a new model of the SOS-response in *E. coli*, which could progresses through three stages (Fig. 8): early period of error-free repair (0–30 min), middle period (30–90 min) that includes Pol IV-mediated TLS, acting at sites aside from replisomes, and mutagenic period (>90 min), when Pol V becomes fully active (Henrikus *et al.*, 2018).

1.3.3.2 Regulation of TLS by general stress response

In addition to SOS response, the expression of *E. coli* Pol IV and Pol II is also under the control of general stress response that is regulated by the alternative RNA polymerase sigma factor RpoS, (σ^S) (Layton and Foster, 2003; Storvik and Foster, 2010; Dapa *et al.*, 2017). This response is activated in bacteria not only when cells are exposed to starvation (or when they enter stationary phase), but also in response to many different stress conditions, such as antibiotics, oxidative damage, osmotic stress *etc.* (Hengge-Aronis, 2002; Bouveret and Battesti, 2011; Gutierrez *et al.*, 2013). The induction of TLS polymerases during long-term stationary phase is required to ensure survival and fitness in nutrient-limited conditions in the absence of exogenous stressors (Corzett *et al.*, 2013). In addition, Pol IV is a component of several cellular responses and is regulated by the heat shock chaperone GroE (Layton and Foster, 2005) and by SOS-independent pathway, induced in a response to inhibition of cell wall synthesis (Pérez-Capilla *et al.*, 2005).

1.3.3.3 Posttranslational regulation of TLS polymerase Pol IV

In addition to the transcriptional level, the activity of TLS polymerases is also regulated through protein-protein interactions (Becherel *et al.*, 2002; Godoy *et al.*, 2007; Cafarelli *et al.*, 2014). Pol IV, the most conserved Y-family polymerase, is present in *E. coli* cells at relatively high basal levels. It was previously estimated that about 250 molecules of Pol IV are present in unstressed cell (compared to 10–20 molecules of replicative Pol III), making it also the most abundant polymerase in cells upon SOS induction (2500 molecules) (McHenry and Kornberg, 1977; Kim *et al.*, 2001; Sutton, 2010). However, recent more sensitive fluorescence microscopy study provided new data, suggesting that in non-stressed bacteria there is only about 20 molecules of Pol IV that rise up to 250 copies upon SOS-induction (Henrikus *et al.*, 2018). Proficient and accurate TLS past certain types of N^2-dG lesions and alkylation damage suggests the importance of Pol IV in TLS past some of the damage that spontaneously arises during the normal cell growth (Jarosz *et al.*, 2006; Bjedov *et al.*, 2007; Kumari *et al.*, 2008; Yuan *et al.*, 2008; Ikeda *et al.*, 2014). Since Pol IV can displace stalled Pol III from the sliding clamp (Indiani *et al.*, 2005; Heltzel *et al.*, 2012),

the possible biological role of Pol IV was proposed to be a "default" TLS polymerase that diminishes disturbances in DNA replication by efficiently recovering stalled replication forks (Kim *et al.*, 2001; Goodman, 2002; Ikeda *et al.*, 2014). However, overexpression of *dinB* results in a mutator phenotype in the absence of any exogenous damage, leads to the inhibition of DNA replication and is lethal to cells (Kim *et al.*, 1997; Wagner *et al.*, 1999; Tang *et al.*, 2000; Kuban *et al.*, 2005; Uchida *et al.*, 2008). Moreover, Pol IV might be partly responsible for the SOS-dependent spontaneous mutagenesis, being involved in the extension of (mis)incorporations introduced by Pol V (Kuban *et al.*, 2006). In addition to its direct role in TLS during DNA replication (replicative or post-replicative TLS), Pol IV promotes error-prone recombination during stress by participating in the repair of double-strand DNA breaks (will be discussed in detail in Chapter 1.5) (Shee, Ponder, *et al.*, 2012; Pomerantz *et al.*, 2013). Moreover, Pol IV acts at stalled transcription complexes, virtually linking TLS with transcription (Cohen *et al.*, 2009, 2010; Cohen and Walker, 2010). Consequently, access of Pol IV to DNA has to be highly regulated to avoid unnecessary mutagenesis. The model of strict Pol IV regulation was recently supported by a single molecule *in vivo* studies, demonstrating relatively low co-localization of the Pol IV next to the replication fork in the absence of DNA damage (Thrall *et al.*, 2017; Henrikus *et al.*, 2018).

The activity of Pol IV is modulated post-translationally through the formation of stable binary and ternary complexes or interactions with several proteins such as RecA, $UmuD_2$ and transcriptional factor NusA (Godoy *et al.*, 2007; Cohen *et al.*, 2009; Cafarelli *et al.*, 2014). Pol IV is likely present in cells mainly in a binary complex with RecA, which enhances both catalytic activity on damaged DNA and fidelity of Pol IV (Cafarelli *et al.*, 2013, 2014). In addition, during the SOS response activation, both RecA and $UmuD_2$ bind to Pol IV and modulate mutagenic potential of Pol IV to generate frameshift mutations (Godoy *et al.*, 2007). Moreover, interaction of Pol IV with RecA was observed *in vivo*, both of which were involved in restoration of stalled replication at the sites of DSBs (Mallik *et al.*, 2015). The above-mentioned role of Pol IV in transcription is mediated by interaction with transcriptional factor NusA, which recruits Pol IV to RNA polymerase encountered lesion-containing gaps, to mediate lesion bypass and gap-filling (Cohen *et al.*, 2009, 2010). Moreover, NusA was shown to be critical for stress-induced mutagenesis mediated by Pol IV (Cohen and Walker, 2010). The activity of Pol IV in *E. coli* is also modulated by polyphosphate kinase (Ppk) that affects activity of Pol IV (Stumpf and Foster, 2005) and by histone-like protein HU, involved in DNA supercoiling, which shapes mutagenic properties of Pol IV during stationary phase mutagenesis by currently unknown mechanism (Williams *et al.*, 2007). Additional insurance against Pol IV-mediated TLS on undamaged templates was found to be associated with reduced ability of Pol IV to use RNA as a primer during DNA synthesis, which is further reduced after RecA binding (Tashjian *et al.*, 2017). Such regulation allows minimizing unwanted access of Pol IV to replication forks on undamaged DNA, protecting genome integrity.

1.3.4 How DNA polymerase selection and exchange occurs?

1.3.4.1 β-clamp as a main control mechanism of translesion synthesis?

β-clamp in bacteria or PCNA in eukaryotes and archaea plays an essential role in the coordination of DNA replication, repair and damage tolerance (Vivona and Kelman, 2003). β-sliding clamp encircles DNA and not only provides binding platform and access to the to the replication fork, but also facilitates processivity of replicative, as well as some Y-family DNA polymerases (Wagner *et al.*, 2000; Heltzel, Scouten Ponticelli, *et al.*, 2009). In addition, it also increases catalytic efficiency of Pol IV in *E. coli* (Bertram *et al.*, 2004). TLS polymerases Pol II, Pol IV and Pol V all require interaction with the β-clamp to mediate their lesion bypass (Becherel *et al.*, 2002; Lenne-Samuel *et al.*, 2002). Despite the fact that the clamp represents the primary interaction site for both TLS and replicative DNA polymerases, the exact role of β-clamp in coordination of these partners is still not understood completely.

All polymerases interact with a hydrophobic cleft located near the C-terminal tail of the β-clamp through a conserved clamp-binding motif (CBM) (Dalrymple *et al.*, 2001). Replicative Pol III and TLS polymerases Pol II and Pol IV all interact with distinct, but overlapping clamp surfaces (Maul, Scouten Ponticelli, *et al.*, 2007). Since β-clamp functions as a homodimer, every clamp contains two such hydrophobic clefts, so it was suggested that it may simultaneously manage two different partners acting as a "tool-belt" (Pagès and Fuchs, 2002). Consistent with the coordinated "tool-belt" model (Fig. 9A), two different polymerases, *i.e.*, replicative and TLS polymerase can bind single β-clamp through contacts with separate hydrophobic clefts simultaneously, and clamp then facilitates their rapid exchange. Such concerted "tool-belt" concept allows rapid switch between replicative and TLS polymerases: when lesion is bypassed, the replicative polymerase changes back, to continue DNA replication (Pagès and Fuchs, 2002; Indiani *et al.*, 2005). However, the structure of the sliding clamp-DNA complex revealed the tilted orientation of DNA molecule as it passes through the β-clamp (Georgescu *et al.*, 2008), with one of the clefts situating closer to the DNA. In this way, the polymerase associated with this cleft would have a priority in controlling the 3′-OH end of the DNA primer (Sutton, 2010; Gabbai *et al.*, 2014).

Another variation of the "tool-belt" model suggests that a single cleft of the β-clamp is able to manage actions of multiple interaction partners on DNA via a CBM-cleft and non-cleft contacts, like LF-cleft rim contact in Pol IV (Fig. 9B) (Heltzel, Maul, *et al.*, 2009). Both contacts were shown to be required for the exchange of the stalled Pol III, but not for the synthesis (Heltzel, Maul, *et al.*, 2009; Heltzel *et al.*, 2012). Recent visualization of reconstructed polymerase exchange model at single-molecule level revealed that during normal growth conditions both Pol IV and Pol III could be bound simultaneously to the β-clamp: with Pol IV at the rim and Pol III on the cleft side, allowing rapid switching with Pol III at the blocking sites (Kath *et al.*, 2014).

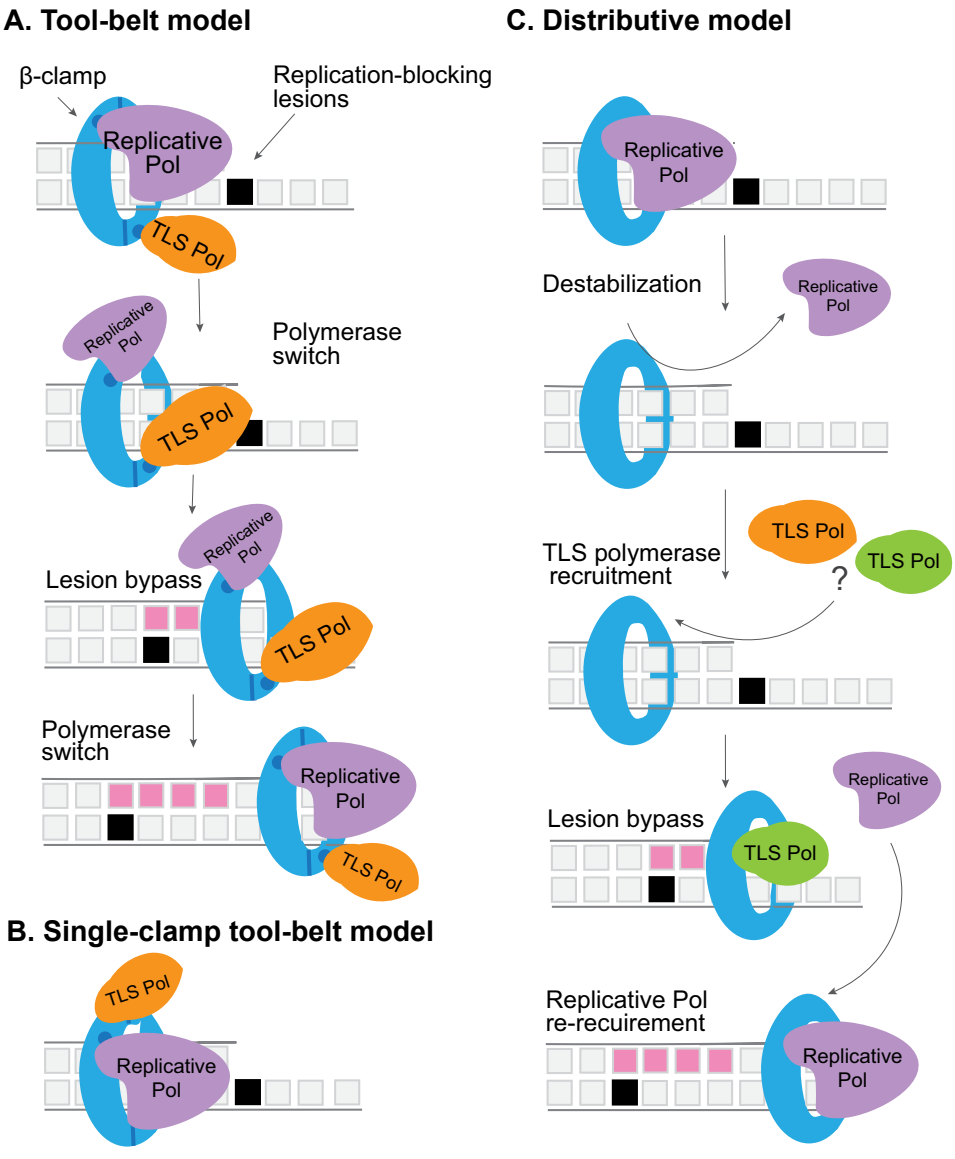

Figure 9. Models for polymerase exchange. (A) In a tool-belt model, replicative and TLS polymerases are bound to separate clefts of the β-clamp. β-clamp mediates the regulation of polymerase switching. Both replicative and TLS polymerases remain associated with the clamp during TLS. When lesion is bypassed, replicative polymerase switches back, and replication continues (β-clamp is indicated in blue; dark blue dots on the β-clamp illustrate the cleft). (B) Alternative single-clamp tool-belt model suggests that both replicative and TLS polymerases are associated with the single cleft of the β-clamp with replicative polymerase through the CBM-clamp cleft interaction and TLS polymerase through a non-cleft surfaces (*e.g.*, Pol IV LF-rim contact). Figure is based on the (Heltzel, Maul, *et al.*, 2009; Sutton *et al.*, 2010). (C) Distributive model of polymerase exchange suggests that replicative polymerase, destabilized by the lesion, dissociates from the β-clamp, allowing TLS polymerase to be recruited to mediate lesion bypass. Based on the (Trakselis *et al.*, 2017).

All these models suggest that TLS occurs at the arrested replication forks and that β-clamp is capable of simultaneous binding of two partners. However, during DNA replication both catalytic and proofreading subunits of Pol III contact β-clamp, and such α-β and ε-β interactions seem to occupy both canonical Pol IV-β-clamp binding pockets, suggesting that Pol IV has no access to the replication machinery (Jergic *et al.*, 2013; Toste Rêgo *et al.*, 2013). Hence, it was suggested that when Pol III is blocked by the DNA lesion in the template, exonucleolytic domain displaces from the clamp due to the weaker affinity, allowing access of Pol IV to mediate lesion bypass (Toste Rêgo *et al.*, 2013). However, recent direct visualization of the replicative Pol III core with Pol II and Pol IV *in vitro*, using co-localization single-molecule spectroscopy, revealed that polymerases compete for binding to the hydrophobic groove of the clamp, and that exchange is rather stochastic and concentration-dependent (Zhao *et al.*, 2017). Moreover, similarly to live cells, where Pol IV predominantly located outside the replisome context, almost no co-localization between Pol III core and Pol IV was observed *in vitro* (Zhao *et al.*, 2017; Henrikus *et al.*, 2018). Therefore, in light of recent findings, the so-called "distributive model" of TLS might exist in *E. coli* cells (Fig. 9C) (Trakselis *et al.*, 2017). The "distributive model" relies on stochastic destabilization of Pol III-β-clamp complex by the obstacle, dissociation from the clamp and the polymerase exchange with partners from the cellular pool, which most likely compete for the β-clamp (Becherel and Fuchs, 2001; Fujii and Fuchs, 2004; Sale *et al.*, 2012; Trakselis *et al.*, 2017; Zhao *et al.*, 2017). Upregulation and increase in concentration of TLS polymerases in response to DNA damage might affect feasibility of lesion bypass (Becherel and Fuchs, 2001; Zhao *et al.*, 2017). The concentration-driven stochastic dissociation and rapid exchange of Pol III* was also recently observed in replisomes during processive DNA replication (Beattie *et al.*, 2017; Lewis *et al.*, 2017). In contrast to Pol III*, β-clamp remained associated with the DNA for a longer time (Beattie *et al.*, 2017), suggesting possibility for other partners to be recruited. Therefore, such stochastic and concentration-driven exchange mechanism would allow frequent but also limited access of different partners to the replisome to bypass or mediate the repair of a lesion (Lewis *et al.*, 2017).

However, what triggers Pol III to dissociate from the clamp? It was suggested that direct interactions between Pol III and Pol IV might promote displacement of Pol III from the clamp (Scotland *et al.*, 2015; Yuan *et al.*, 2016). Yet, no effect of Pol IV binding on Pol III dissociation was observed in recent single-molecule study, but rather two polymerases bound independently and in an alternate way (Zhao *et al.*, 2017). A rapid exchange of Pol IV and Pol III was observed in situations, when Pol III HE encountered a hairpin structure on a template or when Pol III HE collided with the primer/template duplex (Le *et al.*, 2017). The presence of a duplex ahead of the core enzyme might induce rapid internal changes between the polymerase and exonuclease activities of the core, leading to the loss of the contact between DNA and α subunit, resulting in subsequent Pol III-Pol IV exchange. The same hypothesis might also be attri-

buted to polymerase exchange during TLS, when DNA lesion induces not just a simple blockage, but rather "idling" state of Pol III (Le *et al.*, 2017). Taken together, stochastic model of TLS with the polymerase exchange driven by the obstacle, coupled with dynamic and flexible DNA replication model, might be present in *E. coli* cells.

1.3.4.2 Selection of specific polymerase

Many factors were suggested to explain selection and coordination of TLS polymerases inside the cell. Early models proposed the hierarchy between *E. coli* polymerases with the following order during conjugational replication: Pol III > Pol II > Pol IV and Pol V, and Pol III > Pol IV > Pol V>Pol II upon UV-induced damage, suggesting constant interplay between polymerases influencing each others access to the replication fork (Delmas and Matic, 2006; Sutton and Duzen, 2006). However, Pol IV might have priority over other TLS polymerases to access the replication fork, as TLS by Pol II seems to be inefficient in the presence of stalled Pol III (Gabbai *et al.*, 2014; Kath *et al.*, 2016). Moreover, Pol II was shown to exchange with Pol III not within the active replisome, but rather within the released Okazaki fragments on ssDNA gaps (Kath *et al.*, 2016). In addition, the proposed hierarchy order does not reflect the relative concentration of polymerases in a cell. Therefore, another mechanism, such as relative affinity of polymerase to the β-clamp was suggested (Delmas and Matic, 2006). Still, the role of binding affinities of different polymerases in regulation is unclear. Pol II, for example, binds β-clamp almost 3 times more tightly than Pol III, but even in unstressed cells its concentration is 2–3 times higher (Sutton, 2010). The affinity of the particular TLS polymerase to the substrate (nature of a lesion itself) (McCulloch *et al.*, 2004), or selectivity on the location of the adduct in the DNA were also suggested (Fuchs and Fujii, 2013). Pol V in *E. coli* preferably mediates bypass of the lesions located in the major groove (like UV-induced lesions), while lesions located in the minor groove are bypassed by Pol IV (specifically N^2-dG adducts) (Napolitano *et al.*, 2000; Tang *et al.*, 2000; Jarosz *et al.*, 2006; Seo *et al.*, 2006; Yuan *et al.*, 2008; Kath *et al.*, 2014). Such preferential bypass can be attributed to the structural features of TLS polymerases (Chandani *et al.*, 2010). In addition, β-clamp-DNA interactions might also manage the polymerase actions *in vivo* (Heltzel, Scouten Ponticelli, *et al.*, 2009). β-clamp contacts DNA with the same region needed for binding with Pol II and Pol IV. Therefore, it is possible that clamp-DNA interactions may affect the location of the clamp on DNA, determining its governing abilities for different partners (Heltzel, Scouten Ponticelli, *et al.*, 2009).

The simple trial and error mechanism was also suggested: if the particular TLS polymerase fails to mediate the bypass or the TLS patch is too short, the next polymerase will be recruited to restart the process, until the lesion is bypassed (Fuchs and Fujii, 2013). Such trial and error mechanism works well with the "mass-action" controlled stochastic way of TLS observed in *in vitro* condi-

tions, where no effect of lesion specificity on polymerase recruitment was detected (Zhao *et al.*, 2017). However, simple concentration-driven contradicts with recent observations in living cells (Thrall *et al.*, 2017; Henrikus *et al.*, 2018). It appeared that simple increase in cellular concentration of Pol IV was not enough to get access to DNA, so DNA damage, most likely together with other additional factors that accumulate in response to DNA damage, were also required (Henrikus *et al.*, 2018). The recruitment of Pol IV also strongly depended on the type of the damage and multiple interactions with proteins (in addition to β-clamp), location and presence of which might be determined by the lesion specificity (Thrall *et al.*, 2017). Therefore, in living cells the selection of specific TLS polymerase might be much more complex as was thought before, with lesions governing specific polymerase selection through interplay with other proteins present in cells. Thus, emerging single molecule studies and technologies challenge and rewrite previous suggestions and theories, highlighting that at the moment we probably only see the tip of the iceberg of the complex TLS regulations *in vivo*. It will be of great challenge and interest to unravel these critical mechanisms in the nearest future.

1.4 DNA replication in the presence of DNA damage

Transcription machinery, as well as different bound to DNA proteins represent natural replication barriers that cause frequent replication fork pausing in *E. coli* growing under optimal conditions (Merrikh *et al.*, 2011; Gupta *et al.*, 2013; García-Muse and Aguilera, 2016). In addition, replisomes must constantly deal with DNA damage, which results from normal endogenous metabolic processes (De Bont and van Larebeke, 2004). As a consequence, all these obstacles can arrest the progression of replicative DNA polymerases during genome duplication. Due to the asymmetric DNA synthesis, the presence of DNA damage (or other obstacles) in the lagging- and leading-strand has different consequences on DNA replication and affects replication forks differently (Fig. 10) (Higuchi *et al.*, 2003; Heller and Marians, 2006). DNA damage in the lagging-strand is usually efficiently bypassed by bacterial replisomes because of the rapid re-priming for Okazaki fragment synthesis, which allows re-initiation of replication upstream of the DNA lesion on a newly synthesized primer, leaving a small ssDNA gap behind (McInerney and O'Donnell, 2004; Langston and O'Donnell, 2006). Resulting ssDNA gaps, containing DNA lesion, are then repaired post-replicatively (Fig. 10) (Fuchs, 2016).

The fate of the replisome, which encounters an obstacle during leading-strand synthesis, was widely debated (Yeeles *et al.*, 2013). Some models in *E. coli* supported ideas of absolute block to replication, resulting in uncoupling of leading- and lagging-strand synthesis (Pages and Fuchs, 2003; Rudolph *et al.*, 2007). Other theories suggest the model of replisome "skipping" over the obstacle by re-initiation of the synthesis downstream the damage (Fig. 10A), resulting in ssDNA gap that is repaired (filled in) later (Rupp and Howard-

Flanders, 1968; Heller and Marians, 2006; Yeeles and Marians, 2011). Indeed, the replication-blocking lesions, encountered at the leading strand during DNA synthesis (at least temporally), stalls replication fork movement. However, such lesion can be efficiently skipped: replisome can be reassembled downstream of the damage via DnaG-dependent leading-strand re-priming without replisome breakage, leaving ssDNA gaps with damage behind the replication fork (Fig. 10A) (Yeeles and Marians, 2011, 2013). Short ssDNA gaps between the lesion and the 5′-terminus of the growing leading-strand generated this way are filled in either by non-mutagenic RecA-promoted recombination repair mechanism (Fig. 10C) or by the action of TLS polymerases (Fig. 10E) (Berdichevsky *et al.*, 2002; Lehmann and Fuchs, 2006; Bichara *et al.*, 2011; Fuchs, 2016).

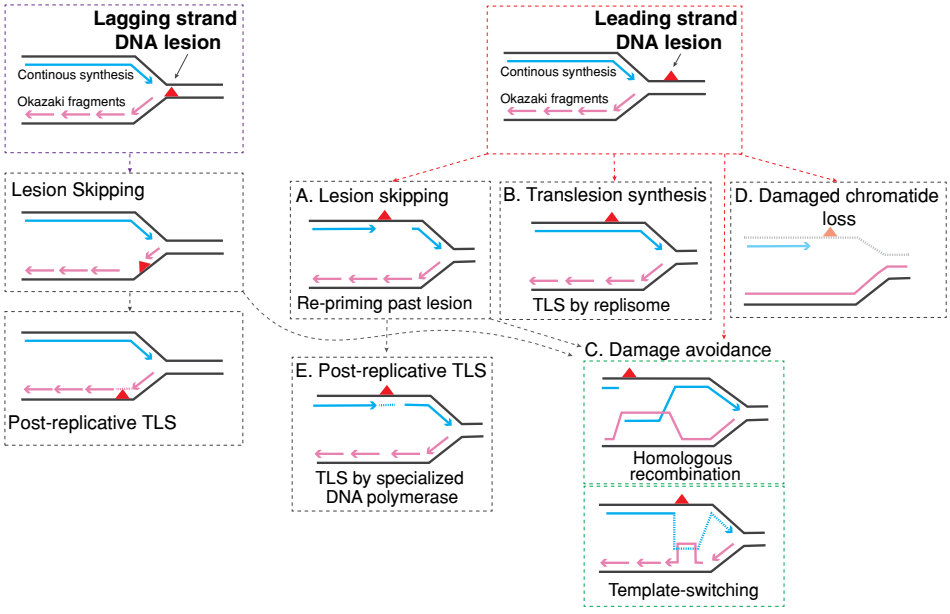

Figure 10. DNA damage tolerance pathways. When replisome encounters DNA lesion (red triangle) during lagging-strand synthesis, it skips the lesion without the replisome blockage. Lesion-containing ssDNA gaps are repaired post-replicatively either by TLS or damage avoidance mechanisms (C). Leading-strand DNA lesion blocks, at least temporarily, fork progression. Replicative polymerase blocking lesion can be either skipped (A) by re-priming downstream the DNA lesion, leaving 1–2kb ssDNA gap, which can be further filled-in by TLS polymerases (E) or repaired via damage avoidance mechanisms (C). Alternatively, damage can be directly tolerated by translesion synthesis (B) by replicative Pol III in a replisome context, or by a fast switching with TLS polymerase, to mediate synthesis of a short TLS patch across and behind the lesion; or by damage avoidance mechanisms (C) like homological recombination or template switch. Damage chromatid loss (D) represents alternative strategy, allowing cell to divide and survive by replicating of only undamaged chromatid. Based on (Laureti *et al.*, 2015; Nevin *et al.*, 2017; Marians, 2018).

Leading-strand lesion skipping is thought to be a competitive process to the replisome-mediated TLS (Fig. 10A, B). It was demonstrated that Pol IV (but not Pol II) was able to mediate leading-strand lesion bypass in the context of the replisome, decreasing the proportion of leading-strand lesion skipping (Gabbai *et al.*, 2014). Alternative model of direct replisome-mediated TLS was also proposed by study demonstrating that Pol III HE in the replisome context was able to replicate past AP sites and cyclobutane pyrimidine dimer located in the leading-strand template (Nevin *et al.*, 2017). The mechanism behind Pol III-mediated TLS is not understood. Upon DNA damage, activation of the SOS response leads to upregulation of dNTP pool levels in bacteria, and this increase in dNTP concentration also somehow strongly promotes Pol III-mediated TLS (Gon *et al.*, 2011). Accordingly, a new potential damage tolerance strategy was suggested: upon DNA damage and under SOS conditions, when nucleotide concentration is increased, Pol III HE in the replisome context might be actually responsible for TLS and mutagenesis that occurs at the replication forks (Fig. 10B). While specialized damage-inducible TLS polymerases act in gaps left behind the lesion resulting from the replisome lesion-skipping (Fig. 10E) (Nevin *et al.*, 2017). Both Pol III HE-mediated TLS and lesion-skipping theories support the observations made in living cells *in vivo*, with Pol IV and Pol V acting outside the replisome context, as was described above (Robinson *et al.*, 2015; Henrikus *et al.*, 2018).

In the excess of DNA damage, when SOS response is activated, increased levels of TLS polymerases Pol II and Pol IV, and RecA restricts and slow down the progression of all replication forks that might be present in rapidly growing and dividing cell (Indiani *et al.*, 2009, 2013; Tan *et al.*, 2015). It was demonstrated that both Pol II and Pol IV were able to form alternative replisomes *in vitro*, which significantly decreased the helicase unwinding speed (Indiani *et al.*, 2009). Moreover, RecA acted as a master switch: stimulated the action of replisomes containing TLS polymerases, while at the same time inhibited the movement of Pol III-containing replisomes (Indiani *et al.*, 2013). Moreover, during early stages of the SOS response, UmuD interacts with the catalytic subunit α and mediates the displacement of Pol III from the β-clamp (Ollivierre *et al.*, 2010; Silva *et al.*, 2012; Murison *et al.*, 2017). Such interaction between UmuD and α specifically inhibits binding of polymerase to ssDNA (Chaurasiya *et al.*, 2013). All these mechanisms provide more time for cell to repair DNA damage, and are possibly needed to avoid potential collisions between transcription and replication machineries in the presence of stress (Duch *et al.*, 2013; García-Muse and Aguilera, 2016).

1.4.1 Postreplicational repair: TLS or damage avoidance?

Both TLS and damage avoidance (DA) mechanisms, such as homological recombination (HR) and template-switching, are the two important DNA damage tolerance (DDT) strategies that allow the cell to duplicate its genome and

survive in the presence of DNA damage (Fig. 10) (Fuchs, 2016). Neither TLS nor DA post-replication mechanisms actually remove replication-blocking lesion, but just temporally bypass it (Lovett, 2017). However, if TLS is inherently error-prone, then the gap repair by more accurate HR or template-switching (Fig. 10C) offers a potential advantage over TLS (Chang and Cimprich, 2009; Fuchs and Fujii, 2013). Thus, the balance and interplay between the TLS and damage avoidance is crucial to keep the mutation load under control. Under physiological conditions, TLS in *E. coli* represents only a minor part (0.5–2%) of the DDT events in comparison with DA, increasing up to 10–20 times upon SOS induction (Berdichevsky *et al.*, 2002; Naiman *et al.*, 2014; Fuchs, 2016). When SOS response is fully induced, it still represents only 30% of overall DDT events, suggesting that in *E. coli* survival is mostly mediated by the DA (Fuchs, 2016). However, under "artificial" overexpression conditions, TLS can be tuned up to 90–100% of events (Naiman *et al.*, 2014).

Defects or inefficiency of homological recombination can lead to the increased TLS (Naiman *et al.*, 2016). Moreover, proximity of replication-blocking DNA lesions can modulate DNA damage response pathways (Chrabaszcz *et al.*, 2018). For example, when lesions are simultaneously present in opposite strands (that can naturally occur under genotoxic conditions), it leads to structural inhibition of homologous recombination upon SOS induction and makes TLS the main DDT mechanism used by the cell (Chrabaszcz *et al.*, 2018). The processing of closely spaced lesions in opposite DNA strands by NER also requires Pol IV/Pol II for the repair of toxic DNA intermediates (Janel-Bintz *et al.*, 2017). Therefore, the lesion structure, location and DNA context can favour particular DDT mechanism to tolerate damage. Following genotoxic stress and SOS induction, TLS in *E. coli* might be the first but also minor DTT strategy (not taking into account primary DNA damage repair mechanisms utilized by cells) that operates before damage avoidance mechanisms take place (Naiman *et al.*, 2014). The duration of TLS phase in cells might depend on the stability of β-clamp, which remains associated with DNA after replicative polymerase dissociation upon encountering DNA lesion, allowing TLS polymerases to be recruited to mediate lesion bypass. When β-clamp dissociates and ssDNA-RecA filaments invade sister chromatids, forming D-loops (HR intermediates), the period of TLS ends, and homological recombination initiates accurate repair of lesion-containing gaps (Naiman *et al.*, 2014, 2016; Fuchs, 2016). Such delay in DA events allows DNA damage to be repaired, but also gives time for TLS to generate enough mutations that enable cells to respond and adapt to stressful conditions (Naiman *et al.*, 2014).

Recently, the existence of a third DTT strategy was demonstrated. Namely, *E. coli* cells were able to divide even in the presence of unrepaired gaps, because of the absence of stringent division checkpoint mechanisms (Laureti *et al.*, 2015). Such strategy allowed bacteria to tolerate DNA damage and survive, but at the expense of a damaged chromatid loss, demonstrating that in bacteria cell proliferation can be favored over faithful DNA repair (Fig. 10D) (Laureti *et al.*, 2015).

Surprisingly, in mammalian cells TLS plays more advanced and critical role than in *E. coli* (Avkin *et al.*, 2004). If the TLS-mediated bypass across an AP site and benzo[a]pyrene-guanine adduct in *E. coli* cells accounted for about 5% and 2% respectively, then in higher eukaryotes TLS reached up to 80% of all damage tolerance events (Reuven *et al.*, 1998; Avkin *et al.*, 2002, 2004; Izhar *et al.*, 2013). In addition, TLS past UV-induced damage in mammalian cells also significantly dominates (89%) over homology-dependent repair, and what is more interesting, this bypass is mostly error-free (Yoon *et al.*, 2010; Izhar *et al.*, 2013). At the same time, in *E. coli* TLS is responsible for the most of UV-induced mutagenesis (Kato and Shinoura, 1977; Kim *et al.*, 1997). Compared to bacteria, eukaryotic cell are well equipped with multiple TLS polymerases, which have evolved to mediate accurate bypass past certain types of cognate lesions, allowing to keep the mutational load at a low level (Livneh *et al.*, 2010; Vaisman and Woodgate, 2017). Therefore, from the genetic point of view, the importance and efficiency of TLS may be directly attributed to the number of TLS polymerases present in the cell (Becherel and Fuchs, 2001; Fuchs and Fujii, 2013).

1.5 The role of TLS in stress-induced mutagenesis

In reality, most of the organisms, especially bacteria, live in constantly changing and stressful conditions. Therefore, the mutation rate for a particular organism actually also depends on the interaction of the environment with the genetic factors and mechanisms that are responsible for DNA damage avoidance, tolerance and repair (Giraud *et al.*, 2001; Massey and Buckling, 2002; Krašovec *et al.*, 2017). Various stressful conditions (starvation, hypoxia, antibiotic exposure) can accelerate mutagenesis in mal-adapted or stressed organisms and potentially drive adaptation (Bjedov *et al.*, 2003; Galhardo *et al.*, 2007; Shee, Gibson, *et al.*, 2012; Matic, 2013; Fitzgerald *et al.*, 2017). The link between stress and mutation can be direct, as DNA damaging agents, present in the environment, can damage DNA and inactivate DNA repair enzymes (Tenaillon *et al.*, 2004). But there is also a view that stress can lead to the increased mutagenesis indirectly, due to the changes in the expression of genes that can modulate or affect the appearance of mutations (MacLean *et al.*, 2013). Stress-induced mutagenesis (SIM) is described as phenomenon that mirrors mutagenesis that is indirectly induced by stress, but not directly mediated (MacLean *et al.*, 2013). The accumulation of Lac^+ revertants during prolonged incubation under selective non-lethal conditions (Cairns and Foster developed system described in Chapter 1.2.1), was interpreted as a direct evidence of stress-induced mutagenesis (Cairns *et al.*, 1988; Cairns and Foster, 1991). Since frameshift mutations occurred under growth limiting (starving) conditions in non-growing bacteria, it was suggested that stress activated processes that led to increased mutagenesis. As such, the stress-induced mutation paradigm assumes that mutation can be under genetic control, and stressful conditions may lead to genetically controlled increase in

mutation rates that can accelerate evolution of antibiotic resistance, pathogen adaptation, as well as tumor progression in human (Galhardo *et al.*, 2007). Such stress-induced mutation mechanism has been observed in different species, including bacteria (Bjedov *et al.*, 2003; Galhardo *et al.*, 2007; Kivisaar, 2010; Shee *et al.*, 2011), yeast (Matsuba *et al.*, 2012) and human (Bristow and Hill, 2008; Roberts *et al.*, 2012). In bacteria, different stresses can regulate mutagenesis via different pathways and mechanisms (Bjedov *et al.*, 2003; Galhardo *et al.*, 2007; Matic, 2017). However, in general, all global stress responses upregulate low-fidelity DNA polymerases and shift to error-prone replication (Ponder *et al.*, 2005), repress DNA repair mechanisms (Feng *et al.*, 1996; Harris *et al.*, 1997; Tsui *et al.*, 1997) and activate movement of mobile elements, consequently increasing genetic variability (Foster, 2007).

The evidences and underlying molecular mechanisms of SIM come from studies with starved *E. coli* cells, demonstrating that stress leads to activation of mechanisms that are actually not essential for proper repair (Ponder *et al.*, 2005; Shee *et al.*, 2011; Rosenberg *et al.*, 2012). Such SIM is associated with mutagenic repair of double-strand breaks (DSB) described in starving *E. coli* cells (Ponder *et al.*, 2005; Shee, Gibson, *et al.*, 2012). In unstressed *E coli* cells, the repair of spontaneously arising DSB via homologous recombination requires Pol III (Motamedi *et al.*, 1999). However, in starving (*i.e.,* stressed, mal-adapted) bacteria, the process of DSB repair becomes mutagenic (Ponder *et al.*, 2005; Shee *et al.*, 2011). The upregulation of RpoS during starvation switches the high-fidelity repair to mutagenic recombination-dependent DSB repair, which is attributed to the error-prone actions of Pol IV (Ponder *et al.*, 2005; Shee *et al.*, 2011). Mutagenic activity of Pol IV was shown to be associated with the DSB repair by mediating the error-prone extension of recombination intermediates, accounting for almost 85% of stress-induced mutations (McKenzie *et al.*, 2001; Pomerantz *et al.*, 2013). Stress-induced DSB-repair-associated mechanism of SIM was observed using different systems, and in addition to Pol IV, Pol II and Pol V were demonstrated to contribute to mutagenesis during starvation (Petrosino *et al.*, 2009; Frisch *et al.*, 2010; Shee *et al.*, 2011). However, upregulation of mutagenic polymerases and DSB repair might not be enough for SIM to happen, as damaged bases in DNA are also needed (Moore *et al.*, 2017). It was recently discovered that under starvation, endogenous ROS-induced 8-OH-dG present in DNA or nucleotide pool is the main driver of spontaneous mutagenic DSB repair. Damage in DNA would trigger TLS polymerase to exchange with stalled replicative Pol III to allow error-prone DSB repair (Moore *et al.*, 2017). Therefore, not only TLS polymerases *per se*, but also spontaneous DNA damage that accumulates in starving bacteria during prolonged incubation are probably responsible for the observed SIM phenomenon.

TLS polymerases also have an important role in evolutionary fitness and long-term survival (Yeiser *et al.*, 2002; Corzett *et al.*, 2013). During long-term stationary phase when incubated separately, the survival of strains lacking TLS polymerases was comparable to that of the wild-type strain. However, in competition with the wild-type strain, *E. coli* mutants lacking even just one TLS

polymerase had lower fitness, and were eventually outcompeted by the wild-type bacteria. Consequently, it was suggested that genetic variation, introduced by TLS polymerases in times of stress was favored, conferring evolutionary fitness to bacteria (Yeiser *et al.*, 2002; Corzett *et al.*, 2013). Therefore, in addition to the direct DNA damage tolerance function the importance of TLS in bacteria can also be related to generation of genetic diversity needed for survival in the time of stressful conditions.

1.6 Strategies of TLS and DNA damage response in other bacteria

Unfortunately, in bacteria, the knowledge about TLS and its function is mostly based on the *E. coli* paradigm. Although *E. coli* serves as a prototype for many studies, the presence of *E. coli umuDC* system responsible for SOS mutagenesis and TLS is rather unique to bacteria (Erill *et al.*, 2006). Diverse classes of bacteria instead of prototypic *E. coli* Pol V actually have an alternative set of genes that is responsible for TLS and induced mutagenesis (McHenry *et al.*, 2011). Damage-regulated *imuA-imuB/dnaE2* multigene cassette was firstly discovered in *Pseudomonas putida* (*P. putida*), representative of Gamma Proteobacteria, in a regulon with a second copy of *lexA* gene*, lexA2* (Abella *et al.*, 2004). Initially, the identified genes in this cassette were annotated as *sulA2* (by homology with *E. coli sulA*)*, dinP* (homologous to *E. coli* Y-family polymerase encoding *dinB/dinP*) and *dnaE2* (homology with *dnaE,* encoding α subunit of replicative Pol III). This *lexA2-sulA2-dinP-dnaE* cassette in *P. putida* is DNA damage-inducible and is regulated by the *lexA2* product (Abella *et al.*, 2004). Subsequently, *sulA2* and *dinP* were renamed after *i*nducible *mu*tagenesis as *imuA* and *imuB* (Galhardo *et al.*, 2005). The product of *dnaE2* was also shown to be associated with mutagenesis in *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Caulobacter crescentus* (*C. crescentus*) (Boshoff *et al.*, 2003; Galhardo *et al.*, 2005; Warner *et al.*, 2010). Therefore, for the clarity, *dnaE2* was also further renamed to *imuC*, to distinguish this homologue from replicative DnaE*,* and while the whole cassette is from now on designated as *imuA-imuB-imuC,* or *imuABC* (McHenry *et al.*, 2011). The homologues and various derivatives of this mutagenic cassette were found to be widely distributed among different bacteria species via vertical inheritance, lateral gene transfer and duplication (Abella *et al.*, 2004; Erill *et al.*, 2006; Zeng *et al.*, 2011; Neus *et al.*, 2012). However, this distribution is very random, as even closely related species might not all possess the *imuC* (*dnaE2*) in their genomes (Timinskas *et al.*, 2014). Because ImuC is frequently present in bacteria with GC-reach genomes, it might have a dominant role in genomic GC-content maintenance (Zhao *et al.*, 2007; Timinskas *et al.*, 2014).

The biological function of the cassette has been elucidated in some bacteria species, however, the individual functions of the components are still not

completely understood. The most comprehensive study, clarifying the roles of individual partners, was performed in mycobacteria (Warner *et al.*, 2010). In *M. tuberculosis,* this SOS-inducible mutagenic operon is composed of *imuA*'-*imuB*/*imuC* genes, and all components of the cassette are essential for the induced mutagenesis and DNA damage tolerance (Erill *et al.*, 2006; Warner *et al.*, 2010). From this trio, only ImuC is an error-prone DNA polymerase that mediates catalytic lesion bypass (Warner *et al.*, 2010). ImuA', despite the structural similarity with RecA or initially suggested homology with *E. coli sulA,* lacks the characteristic C-terminus of RecA, and no self-association was detected in *M. tuberculosis* (Warner *et al.*, 2010). Still, based on the sequence analysis, ImuA' could be a DNA-binding protein, but the exact role of the protein in this "mutasomal complex" is not yet clear. Finally, ImuB, despite high similarity to Y-family polymerases, lacks the conserved catalytic amino acids in the predicted "palm" subdomain critical for polymerase activity (Koorits *et al.*, 2007; Warner *et al.*, 2010). Instead, ImuB possess CBM needed for interaction with β-clamp and interacts with both ImuC and ImuA. Therefore, ImuB most likely has an important regulatory function: it interacts with the clamp and mediates both ImuC and ImuA' to the replication fork, holding "mutasome complex" together (Warner *et al.*, 2010).

This mutagenic cassette is non-essential for survival (Abella *et al.*, 2004; Warner *et al.*, 2010; Tsai *et al.*, 2012). However, *imuABC* genes are required for UV-, and mitomycin C (MMC)-induced damage tolerance in *M. tuberculosis* and *C. crescentus,* and are also responsible for mutagenesis induced by these agents (Galhardo *et al.*, 2005; Warner *et al.*, 2010). In *M. tuberculosis,* the ImuC-mediated mutagenesis is important for adaptation and plays a critical role in the development of antibiotic resistance in mice *in vivo* (Boshoff *et al.*, 2003). Moreover, the moderate but persistent increased expression of *imuC* was observed in some of the rifampicin-resistant *M. tuberculosis* mutants, corresponding to clinical isolates with reduced fitness, suggesting a potential link of ImuC to adaptation of bacteria (Bergval *et al.*, 2007). In *C. crescentus,* however, the artificial expression of the *imuABC* genes at the level comparable to that of the SOS induction, was not mutagenic to cells, suggesting that either the access of the complex to DNA is strictly regulated, or replication on undamaged DNA is error-free (Alves *et al.*, 2017). Moreover, in *Streptomyces*, ImuC was dispensable for UV-resistance and mutagenesis (Tsai *et al.*, 2012). Notably, in two representatives of Pseudomonads, *P. aeruginosa* and *P. putida,* the role of ImuC in UV-induced mutagenesis has been controversial. In *P. aeruginosa,* ImuC has been demonstrated to be responsible for the UV-induced mutations (Sanders *et al.*, 2006); however, ImuC in *P. putida* was shown to have an opposite effect, acting also as an antimutator, reducing the number of base substitutions, during stationary phase (Koorits *et al.*, 2007).

These phenotypic and functional differences observed in different bacteria species could be attributed to the intrinsic specificities of ImuABC, however, this might also reflect innate damage tolerance/repair strategies of the organism. For example, *P. putida,* possess two *lexA* genes, one of them regulating *lexA2*-

imuA-imuB-imuC operon and the second – *E. coli*-SOS-like response, while in *P. aeruginosa*, the mutagenic cassette consists only of *imuA-imuB-dnaE2* genes, being under regulation of *E. coli*-like LexA (Abella *et al.*, 2007). Moreover, these bacteria also differ in the genetic composition of the SOS response, and in the expression of genes responsible for the constitutive DNA damage repair (Cirz *et al.*, 2006; Abella *et al.*, 2007). For example, in *P. aeruginosa* both *uvrA* and *uvrB* that are involved in the repair of different DNA lesions, including those caused by UV light, are induced constitutively (Rivera *et al.*, 1996, 1997). In *P. putida*, only *uvrB* and *uvrD* are induced by DNA damage (Abella *et al.*, 2007). These are just few examples demonstrating that even closely related bacteria like *P. putida* and *P. aeruginosa* may respond differently to DNA damage.

In addition to ImuABC, both Pseudomonads possess DNA-damage inducible Pol IV (DinB) and *polB*-encoded Pol II (Tegova *et al.*, 2004; Cirz *et al.*, 2006; Sanders *et al.*, 2006, 2011). The induction of the *dinB*-encoded Pol IV in *P. aeruginosa* seems to be LexA-independent (Cirz *et al.*, 2006). However, in *P. putida*, Pol IV is under the control of LexA, but its expression occurs at high basal level in cell, being only slightly inducible upon DNA damage (Tegova *et al.*, 2004; Abella *et al.*, 2007). Pol IV in *P. aeruginosa* protects cells against 4-nitroquinilone 1-oxide (4NQO)-induced damage and mediates accurate bypass past 4NQO-, UV-, MMC-induced damage (Sanders *et al.*, 2006, 2011). In *P. putida*, Pol IV is responsible for –1 bp deletion mutations in stationary phase bacteria (Tegova *et al.*, 2004). The role of Pol II in Pseudomonads is not so clear, but in both *P. aeruginosa* and *P. putida*, the expression of *polB* is not detected after exposure of bacteria to ciprofloxacin or MMC, respectively (Cirz *et al.*, 2006; Abella *et al.*, 2007). Finally, one recent study has demonstrated an unexpected role and importance of *P. aeruginosa* Pol II in biofilm formation, with *polB*-deletion affecting twitching and transition of bacteria to the biofilm phenotype (Alshalchi and Anderson, 2014). Therefore, a lot of critical questions about the function, specificity of TLS polymerases in these bacteria, still have no answers, emphasizing the need for further studies.

II AIMS OF THE STUDY

TLS represents an important DNA damage tolerance pathway allowing organisms not only to survive in the presence of DNA damage, but it also constitutes a potential source of mutations that under some circumstances can be beneficial, especially in asexual organisms in the terms of evolution. In bacteria, TLS represents a mechanism through which organism adapts to changing environment, but also develops drug resistance or acquires virulence. Even sub-inhibitory concentrations of antibiotics trigger induction of different stress responses in bacteria, resulting in activation of mutagenic processes, including TLS (Mesak *et al.*, 2008; Gutierrez *et al.*, 2013; Laureti *et al.*, 2013). For example, specialized DNA polymerase Pol IV in opportunistic pathogens *Vibrio cholerae* and *P. aeruginosa* was shown to be one of the key factors responsible for β-lactam-induced mutagenesis (Gutierrez *et al.*, 2013). In another pathogen, *M. tuberculosis,* error-prone TLS polymerase ImuC potentially contributed to antibiotic resistance and virulence (Boshoff *et al.*, 2003). As such, awareness of the process of TLS is of great importance, as it will help us to understand the mechanisms behind the emergence of antibiotic resistance and virulence, making the battle in the future easier.

The aim of this thesis was to elucidate the role of specialized DNA polymerases in mutagenic processes in two representatives of the genus *Pseudomonas, P. putida* and *P. aeruginosa*. For that, we first needed a mutation-detection assay to study spontaneous or damage-induced mutations in growing bacteria. Respectively, the first task of my study was to describe the valid assay that could be used to study mutagenic processes in Pseudomonads. The second aim was to question the role and mutagenic potential of specialized DNA polymerases in spontaneous mutagenesis in *P. putida* wild-type cells and in bacteria lacking DNA Polymerase I (DNA Pol I) functions, and clarify the role of specialized polymerase ImuC in UV-damage induced mutagenesis. The third aim was to explore and elucidate the potential role of specialized DNA polymerases in alkylation damage tolerance in both *P. putida* and *P. aeruginosa.*

III RESULTS AND DISCUSSION

3.1 Molecular characterization of the *rpoB*/Rif^r system to study specificity and underlying mechanisms of mutagenesis in Pseudomonads (Reference I)

Mutation-detection systems allow us to assess and study mutations and unravel the molecular causes and origins of variation. If nowadays affordability of whole-genome sequencing have revolutionized our understanding of mutation and mutation rate estimates, then less than a decade ago, the study of mutagenic processes relied mostly on indirect methods based on different genetic markers in model organisms (Lynch *et al.*, 2016). Still, most of the test systems available and extensively used in *E. coli*, for example, *lacI* forward mutational systems, allowing mutants to use lactose as a carbon source (Sikora *et al.*, 2012), are not suitable to use in other bacteria that are unable to metabolize lactose, including *Pseudomonas* species. Our research group, led by prof. Maia Kivisaar, has previously developed plasmidial test systems to monitor mutagenic processes in starving bacteria, based on the activation of a promoterless phenol degradation operon *pheBA*, permitting bacteria use phenol as a carbon source (Kasak *et al.*, 1997; Tegova *et al.*, 2004). However, mutational processes on extrachromosomal elements and on the chromosome can be different, for example, because of the differences in replication mechanisms and due to the higher copy of plasmids present in cells (Hendrickson *et al.*, 2002; Rodriguez *et al.*, 2002). As such, chromosomal test systems may better represent the molecular events that happen in cell. Still, the number of test systems that can be used to study mutagenic processes in *Pseudomonas* species very limited. Previously, several studies used rifampicin resistance (Rif^r) based *rpoB*/Rif^r test system (Garibyan *et al.*, 2003) for the analysis of mutagenesis in some of the *Pseudomonas* species (Oliver *et al.*, 2000; Meier and Wackernagel, 2005; Sanders *et al.*, 2006; Mandsberg *et al.*, 2009). However, by that time, the rifampicin resistance at the molecular level was only characterized in some of the *Pseudomonas* isolates (Yee *et al.*, 1996; Hosokawa *et al.*, 2002; Meier and Wackernagel, 2005). The *rpoB*/Rif^r system has been previously widely used in *E. coli,* representing a useful instrument for the analysis of mutagenic specificity and underlying mechanisms of DNA damage and repair (Rangarajan *et al.*, 1997; Garibyan *et al.*, 2003; Wolff *et al.*, 2004; Curti *et al.*, 2009).

Rifampicin (Rif) is a broad spectrum antimicrobial, one of the key components of the tuberculosis treatment since late 1960-s (Kerantzas and Jacobs, 2017). The bactericidal activity of Rif comes from its ability to bind RNA Polymerase (RNAP) and inhibit its action (Hartmann *et al.*, 1967). Prokaryotic RNAP core enzyme consist of five subunits: α-dimer (α_2), β, β′ and ω, encoded by *rpoA, rpoB, rpoC and rpoZ,* respectively (Trinh *et al.*, 2006; Conrad *et al.*, 2010). The analysis of the crystal structure of Rif-RNAP complex in *Thermus aquaticus* have demonstrated that Rif binds within the DNA/RNA channel of the β subunit,

leading to the blockage of the RNA elongation when the transcript is 2–3 nucleotides long (Campbell *et al.*, 2001). Rifampicin does not block the catalytic site nor interfere with substrate binding. Besides, RNAP becomes resistant to Rif after it has synthesized transcript longer than 3–4 nucleotides or entered elongation phase (Campbell *et al.*, 2001). Rifampicin target, the β subunit, is highly conserved among prokaryotes (Musser, 1995; Campbell *et al.*, 2001), and the analysis of Rif^r mutants across different species has demonstrated that Rif resistance results from the mutations in the *rpoB* gene leading to amino acid exchanges in the encoded protein (Garibyan *et al.*, 2003; Conrad *et al.*, 2010). These residues locate either next to the binding pocket or make direct interaction with the drug, and mutations resulting in amino acid substitutions at these positions affect conformation of the binding pocket or lower binding affinity of the antibioticum (Campbell *et al.*, 2001). High level of conservation of key regions of β subunit makes the *rpoB*/Rif^r system affordable to be used in different bacteria species to study mutagenic processes (Garibyan *et al.*, 2003).

3.1.1 Phenotypic heterogeneity of Rif^r mutants

To investigate spontaneous mutagenesis and determine spontaneous mutation frequency in *P. putida* and *P. aeruginosa* we performed fluctuation test and selected mutants on plates containing rifampicin. During the selection period, we observed a diversity in the size of the colonies emerging on Rif-containing agar plates. After 24 h of incubation, plates contained clearly visible large colonies and some almost undetectable small colonies, which became clearly visible, or only appeared after 48 h of incubation. Variability in the size of colonies on Rif containing plates was observed also in other studies (Garibyan *et al.*, 2003; Zeibell *et al.*, 2007), and to avoid the bias, size of the colony was usually invalidated to select mutants for analysis. However, we decided to take a closer look at this phenomenon, and divided Rif^r mutants based on the colony growth parameters into two groups: "large/fast" that emerged 24 h after plating, and "small/slow" that appeared 48 h after plating, and analyzed the frequencies separately. Based on this grouping, the median frequencies of the appearance of "large" and "small" colonies for *P. aeruginosa* were 1.6×10^{-9} and 3.4×10^{-9}, and for *P. putida* 1.9×10^{-9} and 5.7×10^{-9}, respectively, demonstrating that the number of spontaneous mutations is almost similar and general for both Pseudomonads, with surprisingly higher number of mutants appearing on the second day.

To understand the difference in the appearance of small and large colonies, we picked a number of independent Rif^r mutants that emerged on the first and second days after plaiting and plated appropriate dilutions of cells again on plates containing rifampicin. The appearance of the "small" colonies was delayed, if to compare to the appearance of the "large" ones, suggesting that the difference in the emerging time could be attributed to a slower growth of "small" Rif^r mutants in the presence of antimicrobial. In the absence of rifampicin, no significant difference was observed. In addition, we tested the susceptibility of some of the

P. aeruginosa Rif^r mutants to the increased concentrations of antibiotic. The minimal inhibitory concentration (MIC) of the rifampicin for the tested "large/fast" mutants was higher than for the "small/slow" ones (MIC was in the range 900–1600 μg/ml and 100–700 μg/ml, respectively), demonstrating that colonies that appeared on the first day after plating also tolerated higher concentrations of rifampicin. Based on these phenotypic characteristics we classified "large/fast" colony variants as mutants with "strong Rif^r phenotype" and "small/slow" colony variants as mutants with the "mild Rif^r phenotype". Subsequently, we analyzed the distribution of mutations in the *rpoB* in these two groups separately.

3.1.2 Mutations that confer strong and mild Rif^r phenotypes cluster into different groups

The majority of mutations that confer Rif^r in different bacteria species map to four different regions of the *rpoB* gene, referred to as N-terminal cluster, and clusters I, II, III (Fig. 11A) (Jin and Gross, 1988; Severinov *et al.*, 1993, 1994; Campbell *et al.*, 2001; Garibyan *et al.*, 2003). These regions are highly conserved across prokaryotes, and mutations that yield Rif^r phenotype fall into the corresponding residues in the homologous region of *E. coli* of the *rpoB* gene (Fig. 11B). Comparison of the deduced amino acid sequences of RNA polymerase β subunit from *P. putida* KT2440 and *P. aeruginosa* PAO1 strains with this of the *E. coli* K-12 strain MG1655 revealed the high degree of conservation of the corresponding clusters. The N-terminal cluster, and the clusters II and III are identical between species, and only one amino acid from the cluster I differs between *E. coli* and Pseudomonads studied (I530 in *E. coli,* that corresponds to V535 in *Pseudomonas* species) (Fig. 11B).

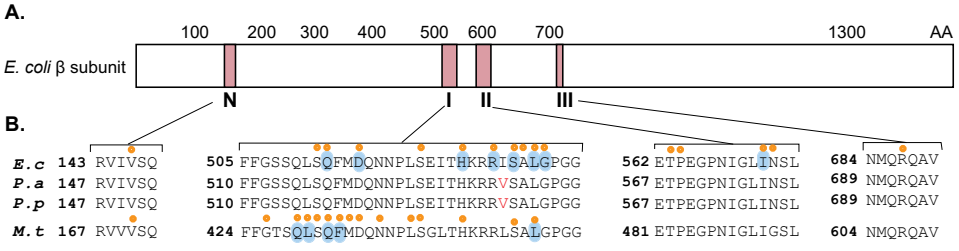

Figure 11. Graphical representation of *E. coli* RNA polymerase β subunit (A) and sequence alignment of the conserved regions of *E. coli* (K-12, substrain MG1655; *E.c*), *P. aeruginosa* (PAO1 strain, locus PA4270; *P.a.*), *P. putida* (KT2440, locus PP0477; *P.p*) and *M. tuberculosis* (*M.t*) (B). (A) Schematic representation of the *E. coli* RNA polymerase β subunit, with amino acid (AA) numbering indicated above. Red boxes indicate clusters (N-terminal cluster, I, II and III), where mutations leading to Rif^r were detected in *E. coli* and/or *M. tuberculosis.* (B) Amino acids colored in blue are in direct contact with Rif; orange dots indicate amino acid substitutions that are associated with Rif^r in *E. coli* and *M. tuberculosis.* Based on (Campbell *et al.*, 2001).

Analysis of the spectra of Rif^r mutants has revealed that mutations that confer strong and mild Rif^r phenotypes were clustered into different groups (Table 2; Ref. I; and Fig. 12 in this thesis), suggesting that specific mutations in the *rpoB* gene might be responsible for the observed phenotypic differences. In *P. aeruginosa*, all mutations that yielded strong Rif^r phenotype were distributed across the I cluster with a mutational hotspot at 1562 site with A:T→G:C change (33 occurrences out of 67). The same hotspot site was also observed in *P. putida* (A:T→G:C; 63 occurrences out of 93). However, to our surprise, this mutation conferred mild Rif^r phenotype in *P. putida*. Mutations yielding mild Rif^r phenotype in *P. aeruginosa* were distributed more randomly and involved multiple substitutions and 2 insertions across cluster I, four substitutions at three sites within cluster II; and 2 substitutions at 2 sites in a region located between cluster II and III. Interestingly, almost 2/3 of the sequenced *P. aeruginosa* mutants with mild Rif^r phenotype did not contain any nucleotide change in the region of the *rpoB* gene encompassing these three clusters. The additional sequencing of the N-terminal cluster of 40 mutants has revealed that 32 mutants had A:T→T:A transversion and 6 A:T→G:C transition at the position 455 within this region of the *rpoB* gene. Still, for two mutants no changes were detected. As such, our data demonstrates that in *P. aeruginosa,* majority of mutations conferring mild Rif^r phenotype map to the N-terminal cluster of the *rpoB* gene. In contrast to *P. aeruginosa*, all mutations accounting for the mild Rif^r phenotype in *P. putida* were mapped to the cluster I, with two significant hotspots: A:T→G:C at 1562 site, as mentioned above, and the second A:T→G:C change at 1592 site, accounting for 18 mutations out of 93 analyzed. The strong Rif^r phenotype in *P. putida* was associated with mutations also located exclusively in cluster I: with significant hotspots at 1553 site, with A:T→T:A change (19 occurrences) and A:T→G:C (14 occurrences), which was not detected in *P. aeruginosa*; and at the position 1607 (C:G→T:A; 16 occurrences). The C→A change at the position 1612 observed in *P. putida* mutants with strong Rif^r phenotype was also unique to this organism. Consequently, considering the phenotypic and mutational data together, we can assume that various mutations in the *rpoB* gene confer differential resistance to rifampicin and have different effect on the growth of bacteria in the presence of the antimicrobial compound.

3.1.3 Growth temperature affects the mutational signatures of bacteria

As mentioned above, the mutational signatures yielding Rif^r phenotypes (especially mild phenotypes) in *P. aeruginosa* and *P. putida* strains were different (Table 2; Ref. I). Out of the 39 mutations detected across the clusters I–III of the *rpoB* gene, 22 were found only in *P. aeruginosa* and nine mutations only in *P. putida.* Such difference could be attributed to the specificities in mechanisms that generate and avoid mutations (for example, replication and repair) in two bacteria species. However, as the growth and selection of

P. aeruginosa and *P. putida* Rif^r mutants was performed accordingly to their optimal growth temperatures, at 37 °C and at 30 °C, respectively, we suspected that such difference in the spectra could be associated with the growth temperature. Previously, temperature-sensitive Rif^r mutants have been described in *E. coli* (Reid, 1971; Jin and Gross, 1988; Garibyan *et al.*, 2003). For example, Garibyan *et al.* have detected that some of the Rif^r mutants isolated at 37 °C failed to grow in the presence of rifampicin when incubated at lower temperatures (Garibyan *et al.*, 2003).

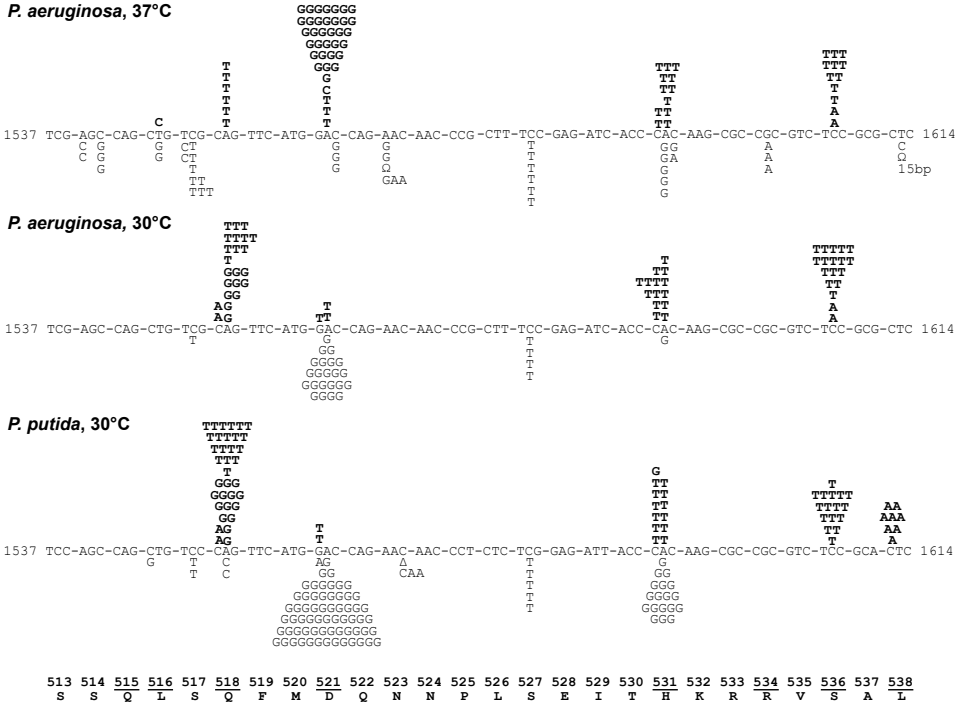

Figure 12. Mutations that confer Rif^r in Pseudomonads within the cluster I of the *rpoB* gene (Related to Table 2; Ref. I). Strong Rif^r phenotype yielding mutations are indicated above in bold and mild Rif^r phenotype below the sequence in *P. aeruginosa* isolated at 37 °C and 30 °C, and in *P. putida* at 30 °C, as follows: Ω – for insertions, Δ – for deletions. Corresponding to *E. coli* and *T. aquaticus* (Campbell *et al.*, 2001) amino acids of RNAP β subunit directly interacting with Rif are shown underlined.

To study the effect of incubation temperature on the selection of mutants, we examined the frequency and the nature of mutations in *P. aeruginosa* Rif^r mutants grown at 30 °C. To our surprise, the analysis of the spectrum and the frequencies of mutations derived in *P. aeruginosa* strain incubated at this temperature revealed a remarkable similarity to that observed in *P. putida* (Table 2; Ref. I; Fig. 12 in this thesis). In particular, the A:T→G:C transition at the position 1553, which was not previously observed in *P. aeruginosa* at

37 °C, was detected in mutants selected at 30 °C as an important hotspot (10 out of 55 analyzed). In addition, the A:T→G:C transition at the position 1562, which was a hotspot in *P. aeruginosa* Rif^r mutants yielding strong phenotype at 37 °C, was observed among mutants that appeared on the second day after plating at 30 °C, similarly to *P. putida*. What was more interesting, if at 37 °C almost 2/3 of the mutations that yielded mild Rif^r phenotype in *P. aeruginosa* located outside of the region encompassing clusters I–III, when incubated at 30 °C, 30 mutations out of 33 analyzed were found to be present within this region. Thus, this data clearly demonstrates that incubation temperature of bacteria affects the spectra of spontaneous mutations conferring Rif^r in the *rpoB* gene in *Pseudomonas* species.

Moreover, some of the *P. aeruginosa* Rif^r mutants expressed so-called "cold-sensitive" phenotype. We compared the growth of three mutants with substitutions located in the I-cluster of the *rpoB* gene (L516P, L516R, H531Q), mutants with substitutions within the N-terminal region and two mutants with unknown mutation which were initially isolated at 37 °C (but not detected at 30 °C) at different temperatures. The appearance of colonies of these mutants on plates containing rifampicin (except for L516P mutant) at 30 °C was delayed for at least for 2 days, if compared to plates incubated at 37 °C, demonstrating strong effect of the temperature on the growth of Rif^r mutants. Notably, additional analysis of mutants that appeared on Rif-containing plates at 30 °C after 3–6 days of incubation has revealed several mutations that were not previously observed during the first two days of selection, including those that occurred in the N-terminal cluster of the *rpoB* gene. Taken together these results demonstrate that slower growth of Rif^r mutants on Rif-containing plates at lower temperature could be associated with the specificity of mutation, explaining why some of the mutations were not detected (or underrepresented) during the screening.

What is the reason of such growth difference? It is not a surprise that acquisition of antibiotic resistance as a result of mutation comes with a cost in a relative fitness of bacteria (Vogwill and MacLean, 2015). Rifampicin targets enzyme essential to life, as prokaryotes have and use only one RNAP to produce all necessary mRNAs, rRNAs and tRNAs (Archambault and Friesen, 1993). Mutations that confer Rif resistance can alter the proper functioning of the RNAP and compromise global gene transcription, and consequently affect physiology, metabolism and overall fitness of bacteria (Jin and Gross, 1988; Reynolds, 2000; Wichelhaus *et al.*, 2002; Mariam *et al.*, 2004; Alifano *et al.*, 2015). The results of our study, as well previous studies with *E. coli* Rif^r mutants (Jin and Gross, 1989), demonstrate that some mutations in the *rpoB* gene may confer slow-growth and/or temperature sensitive phenotypes. As mutations in the *rpoB* can lead to improper folding and/or functioning of the RNAP, or affect interactions with other elements, including sigma factors (Jin and Gross, 1989), specific mutation might have pleiotropic effect on growth (phenotype) at different temperatures. In addition, as effects of mutation on the growth were

observed in the presence of antimicrobial, the possible inhibiting effect of Rif on the appearance of colony might also be present.

The use of comparative technologies has demonstrated and confirmed that *rpoB* mutations underlying Rif^r have a variety of complex and differential effects on the global gene transcription exerting variable effects on phenotypes (Neri *et al.*, 2010; Bisson *et al.*, 2012; de Knegt *et al.*, 2013). For example, certain *rpoB* mutations in *M. tuberculosis* triggered compensatory transcriptional changes in specific secondary metabolites (Bisson *et al.*, 2012). In addition to compensatory transcriptional changes, the presence of compensatory mutations in *rpoA* and *rpoC* genes encoding α and β′ subunits of RNAP, respectively, was detected (Comas *et al.*, 2011). Common trends were also observed among clinical Rif^r *Salmonella enterica* isolates, where acquisition of secondary mutations in *rpoA* and *rpoC* genes enhanced the growth of some slow-growing mutants and reduced fitness cost associated with Rif^r mutations in the *rpoB* (Brandis *et al.*, 2012; Brandis and Hughes, 2013). As such, the molecular mechanism underlying rifampicin resistance and survival can be much complex (Koch *et al.*, 2014).

If we go back to the temperature effect on mutation, then comparison of the spectra of spontaneous mutations in two different *Pseudomonas* species revealed also interesting mutagenic features of bacteria. Relatively similar hotspots and patterns of spontaneous mutations observed in two different representatives of Pseudomonads incubated at one temperature suggests that mutagenic processes (or specificities and activities of polymerases and repair proteins) of these organisms could be similar. Although, we cannot exclude the effect of the temperature on thermodynamics of DNA or/and thermodynamic interactions of DNA with proteins involved in replication and repair (Yakovchuk *et al.*, 2006; Driessen *et al.*, 2014). Temperature can affect DNA duplex structure and local DNA topology, influencing the distribution of endogenous DNA damage, important determinant of spontaneous mutations (Maki, 2002). Considering all the data together, we can conclude that incubation temperature of Pseudomonads has an important impact on the distribution of spontaneous mutations in the *rpoB* gene. In addition to the temperature-dependent DNA topology and DNA-protein transactions, the pleiotropic effects of *rpoB* mutations at different growth conditions can also influence the specificity of mutational spectra.

3.1.4 How suitable is *rpoB*/Rif^r assay for the study of mutagenic processes in Pseudomonads?

The most important findings of our study is that the day of isolation of the mutant (i) and incubation temperature (ii) actually can dramatically affect the overall specificity of spontaneous mutations identified in the *rpoB* gene (Table 3; Ref. I). For example, the analysis of the spectra of Rif^r *P. aeruginosa* mutants selected on the 1-st day after plating demonstrates the prevalence of A:T →G:C transitions (51%), followed by A:T→T:A transversions (28%) and G:C → A:T

changes (16.5%), with none G:C→C:G identified. However, on the second day, mutants with G:C→A:T transitions dominate (40%), with none of the A:T→T:A transversions detected in the region encompassing clusters I–III. All A:T→T:A transversions that appeared on the second day after plating were detected in the N-terminal region of the gene. The temperature adds additional variable to this analysis. The pattern of mutations from *P. aeruginosa* Rif^r mutants selected at 30 °C on the 1-st day after plating demonstrates the G:C→A:T prevalence (38%) with almost the same proportion of A:T→T:A transversions (35%). However, the high frequency of A:T →G:C transitions (77%) detected in mutants that appeared on the second day after plating overrule the mutation specificity and introduce the A:T →G:C bias to the overall mutational spectrum. It is interesting, but in *P. putida* (selected at 30 °C) the spontaneous mutations appear at quite similar proportions to that observed in *P. aeruginosa* selected at 30 °C. With G:C→A:T transitions (30%) and A:T→T:A transversions (34%) prevailing at the first day, followed by the drastic increase in the frequency of A:T →G:C on the second day (88%), resulting in the overall mutational bias towards A:T →G:C mutations. Despite the differences in the mutation types observed at two different days, we still would suggest to select and analyze mutants to study mutagenesis that have appeared at least two days after plating, as different mutagenic processes can lead to the appearance of specific mutations. Due to the possible effect of the mutation in the *rpoB*, affecting the growth of bacteria, selection of mutants only at the first day after plating can underestimate certain types of mutations.

Whole genome analysis of *P. aeruginosa* MA lines revealed strong mutational bias towards the G:C→A:T transitions (Dettman *et al.*, 2016). However, our results based on the *rpoB*/Rif^r analysis demonstrate that A:T →G:C transitions prevail (Table 3; Ref. I). The overall A:T→G:C bias observed in the mutation spectra is associated with a mutational hotspot at the 1562 position in the *rpoB* gene, detected both in *P. aeruginosa* (at both temperatures) and *P. putida* Rif^r mutants. The corresponding D516G is also a mutational hotspot in *E. coli* (Garibyan *et al.*, 2003). As was reviewed in Chapter 1.2.3.1, local sequence context can influence the fidelity of DNA polymerases, and nucleotides that are flanked by a G:C pair mutate at higher rate (Dettman *et al.*, 2016). Mutating A nucleotide at the position 1562 is flanked with 5′-G and C-3′ (5′-G**A**C-′3), which was shown to have one of the greatest mutation rates among the possible triplet combinations (Dettman *et al.*, 2016). Other important hotspots observed in *P. aeruginosa* and *P. putida* were associated with the 5′-C**A**G-‘3 triplet combination (at 455 and 1553 sites), and 5′-C**A**C-′3 (at 1592 position), supporting the context-dependent mutation rates observed in previous studies (Garibyan *et al.*, 2003; Lee *et al.*, 2012). As such, the presence of highly mutable sequence context in the reporter gene can dramatically affect the whole spectra of mutations. In addition, the C→T transitions at the position 1607 underlie the mutational hotspots in both *P. putida* and *P. aeruginosa* (at both temperatures). Such mutation type can be linked to a spontaneous hydrolytic conversion of cytosine

to mutagenic uracil that pairs with adenine leading to the conversion of G:C into A:T (Lindahl, 1993).

Our results are in a good concordance with a recent study in *P. aeruginosa*, where analysis of mutations in the *rpoB* gene also revealed the A:T→G:C hotspot at the 1562 site, with the overall A:T→G:C transition rate of 41% and 30% for G:C→A:T type (Monti *et al.*, 2013). In addition, the authors of this study described novel chromosomally encoded *nfxB*/Cip^r reporter system for mutation analysis in *P. aeruginosa* (Monti *et al.*, 2013). The *nfxB*/Cip^r allowed to detect different mutagenic processes, and the overall spectrum of mutations was composed of 53% of base substitutions, 19% of 1-bp insertions and deletions, 18% of deletions longer than 1-bp, and 8% of duplications. The spectrum of base substitutions was slightly different form that identified by the *rpoB*/Rif^r system: despite the presence of 33% of G:C→A:T transitions from the overall number of substitution mutations, almost 39% were A:T→C:G transversions, due to the hotspot bias associated with the preferred sequence context in the reporter loci. Therefore, if to compare genome-wide analysis data available today (Dettman *et al.*, 2016), the use of reporter genes as mutational markers might have some limitations due to the presence of hotspot sites. Moreover, as β-subunit is required for RNAP function, important classes of mutations, including frameshifts, big deletions or duplications, generally cannot be detected (the exception are in-frame deletions and duplications). In addition, only a limited number of mutable sites (mutations) conferring Rif^r, may lead to underestimation of the overall amount of mutations. Despite these limitations, the *rpoB*/Rif^r system still represents an affordable and reliable marker to study mutagenic processes (spontaneous and induced) in various bacteria species (Garibyan *et al.*, 2003; Baltz, 2014).

In conclusion, the results of our study extended the knowledge about the *rpoB*/Rif^r mutational system and validated its specificity in Pseudomonads (Fig. 13). We demonstrated that the *rpoB*/Rif^r can be used in *Pseudomonas* spp. to study mutagenic processes that lead to alterations in base substitutions. Consistently with the crystallographic data, rifampicin resistance in *P. aeruginosa* and *P. putida* was mostly associated with the mutations leading to substitutions of residues that are involved in direct interactions with Rif or important for the formation of the Rif binding pocket. Due to the presence of highly mutable sequence contexts (mutational hotspots) in the gene, the A:T →G:C and G:C→A:T transitions appear most frequently. As such, the translation of the results from the *rpoB*/Rif^r assay for the whole genome must be done with caution. In addition, our data revealed that the growth temperature of bacteria and time of the mutant isolation must be taken into account, as these factors can influence the number and especially specificity (nature) of mutations. This can be specifically important when the *rpoB*/Rif^r system is used to analyze mutagenic processes in different *Pseudomonas* species that are usually cultivated under different temperature regimes.

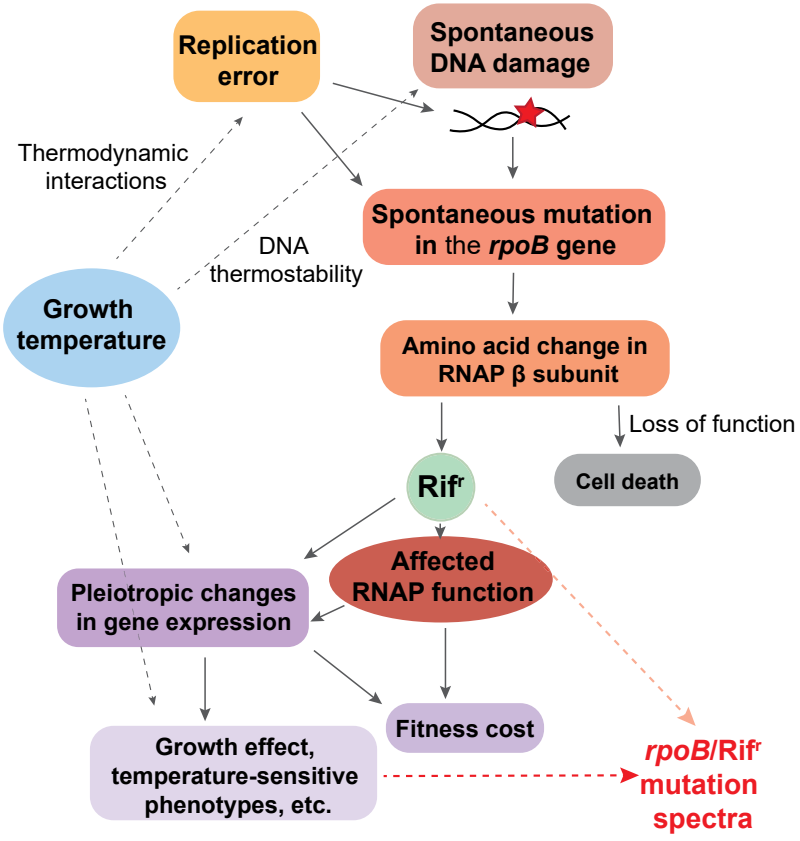

Figure 13. Rifampicin resistance and specificity of the *rpoB*/Rif^r mutation spectra in *Pseudomonas* species. Graphical representation of factors affecting mutation spectra. Specificity of the *rpoB*/Rif^r test system is not as strait forward as it seems to be. Spontaneous DNA damage and replication errors can lead to mutations in the *rpoB* gene resulting in Rif^r. However, some Rif^r mutations in the *rpoB* gene can influence RNA polymerase (RNAP) function, affect the growth of bacteria and, therefore, influence the specificity of the spectra. Growth temperature adds additional variable to the mutation specificity, as it can directly affect DNA thermostability, influencing the appearance of spontaneous DNA lesions, or affect DNA-protein thermodynamic interactions, that can all influence the spontaneous mutation specificity and location. In addition, temperature can affect specificity indirectly, due to the pleiotropic effects of *rpoB* mutation (on gene expression, growth, or on demands for the RNAP at different growth temperatures), affecting the growth of bacteria in the presence of antimicrobial and, therefore, mutant selection.

3.2 Defining the roles of TLS polymerases in *P. putida* in DNA replication (Reference II)

Errors that arise during DNA replication are the major source of spontaneous mutations in cells (Fijalkowska *et al.*, 1993). The majority of chromosomal DNA synthesis in *E. coli* is mediated by the high-fidelity replicative Pol III, with Pol I playing an important role in the synthesis of the lagging-strand being involved in Okazaki fragment maturation (Okazaki *et al.*, 1971; Friedberg *et al.*, 2006). Other polymerases, Pol II, Pol IV and Pol V, found in *E. coli*, are not directly involved in chromosome replication. However, since their discovery, it was suggested that due to the inherently error-prone nature, they might be responsible for a part of spontaneous mutations that arise in cell (Strauss *et al.*, 2000), and indeed, several studies demonstrate that TLS polymerases might have access to DNA during replication (Strauss *et al.*, 2000; Gawel *et al.*, 2008; Curti *et al.*, 2009). For example, Pol IV, which is present at relatively high basal levels in normally growing cells, might be involved in gap-filling, especially during synthesis on the lagging-strand (Kuban *et al.*, 2005). In addition, Pol IV might mediate TLS past constantly arising endogenous lesions (Bjedov *et al.*, 2007). Pol II, due to the presence of proofreading activity, might be involved in the repair of the misinsertion errors on the lagging-strand introduced by replicative Pol III during chromosome replication and in preventing access of Pol IV to the 3′ mismatched termini (Cai *et al.*, 1995; Banach-Orlowska *et al.*, 2005; Gawel *et al.*, 2008). Consequently, constant interplay between polymerases might have an impact (positive or negative) on the occurrence of spontaneous mutations and the genome integrity even under normal growth of bacteria.

The genome of *P. putida* encodes for thee TLS polymerases: (i) *dinB*-encoded PoI IV, representative of Y-family polymerases; (ii) *polB*-encoded Pol II; and (iii) ImuC (former DnaE2, a paralogue of *E. coli* DnaE, the catalytic subunit of Pol III), which is a component of DNA-damage inducible *lexA2-imuA-imuB-imuC* cassette (Abella *et al.*, 2004; Tegova *et al.*, 2004; Koorits *et al.*, 2007). Differently from *E. coli,* the expression of Pol II in *P. putida* is not DNA damage-inducible (Abella *et al.*, 2007). In addition, the transcription from the *dinB* promoter occurs in *P. putida* at high basal level (Tegova *et al.*, 2004; Abella *et al.*, 2007). Therefore, the basal levels of TLS polymerases in *P. putida* cells might be higher. Hence, the next step in my work was to analyze the role of TLS polymerases in spontaneous mutagenesis in *P. putida*, examine their function in bacteria lacking DNA polymerase I (Pol I) and clarify the conflicting role of ImuC in the UV-induced mutagenesis (Reference II).

3.2.1 TLS polymerases leave mutagenic fingerprints

To study the potential role of TLS polymerases in *P. putida* in generation of spontaneous mutations during normal growth of bacteria, we analyzed the frequency and distribution of mutations in the *rpoB* gene in strains deficient

either in Pol II, Pol IV or ImuC (strains PaWPolB, PaWDinB and PaWDnaE2, respectively, in Ref. II) and compared this to a previously described wild-type spectrum (Ref. I). Our results demonstrated that deficiency in any of the specialized polymerase in *P. putida* had no effect on the spontaneous mutation frequencies (Tables 4 and 5; Ref. II), suggesting that TLS polymerases do not contribute to the spontaneous mutagenesis in *P. putida* cells. However, comparison of the spectra in mutants deficient in specialized TLS polymerases with that of the wild-type strain has revealed some differences in the distribution of mutations at specific sites within the *rpoB* gene (Table 4; Ref. II). The biggest difference in the mutagenic events was observed in the strain lacking Pol IV (PaWDinB strain) (Table 4; Ref. II). We observed a significant increase in the C→T transitions at the positions 1550 and 1580 of the *rpoB* gene. In both cases, C was mutated into T within 5′-T<u>C</u>-3′ sequences, suggesting the involvement of Pol IV in suppression of mutagenesis at certain DNA sequence context. In addition, there was a drastic increase in the A→G transitions at the position 1553 (5′-C<u>A</u>G-3′) that was accompanied with simultaneous decrease in the same mutation type, but in a different sequence context (5′-G<u>A</u>C-3′) at the 1562 position of the gene. Interestingly, this A→G mutation at the position 1562, almost undetected in bacteria lacking Pol IV functions, was an important hotspot in the wild-type bacteria (Table 4; Ref. II). As such, Pol IV might be responsible for the majority of A:T→G:C transitions in the wild-type bacteria that occur within the 5′-G<u>A</u>-3′ sequence context. In support of these findings, similar sequence specificity of Pol IV-induced A:T→G:C transitions was observed also in *E. coli,* where Pol IV was responsible for about 70% of 5′-G<u>X</u>-3′ substitutions where X was mutated into G (Wagner and Nohmi, 2000). It was demonstrated that Pol IV might be involved in mutagenic bypass past endogenous ROS-induced damage, enhancing the appearance of A:T→G:C mutations (Hori *et al.*, 2010). Taken together, our data revealed the mutational specificity of Pol IV, implying that Pol IV in *P. putida* may have considerable access to DNA replication, influencing the mutagenic load not only within the *rpoB,* but probably also across the whole genome.

3.2.2 Both Pol II and Pol IV are involved in DNA replication in the absence of Pol I in *P. putida* cells

DNA Pol I is a multifunctional enzyme in cells: it removes RNA primers that initiate Okazaki fragments and replaces with dNTPs during lagging-strand DNA synthesis (Okazaki fragment maturation). Moreover, Pol I plays an important role in DNA repair being involved in re-synthesis step during the NER, BER and recombination (Okazaki *et al.*, 1971; Lehman, 1981; Friedberg *et al.*, 2006). Encoded by a *polA* gene, Pol I has two intrinsic functional domains: the Klenow domain that possesses both 5′→3′ polymerase and 3′→5′ proofreading exo-nuclease activities that edits 3′ mismatches in the nascent DNA, and 5′→3′

exonuclease domain that mediates the removal of RNA primers or nucleotides from the 5′-end (Mizrahi *et al.*, 1986). The growth of *E. coli* lacking Pol I functions is strongly impaired in the rich medium, but the viability of the mutants can be restored when either of the exonuclease fragments is provided *in trans* (Joyce and Grindley, 1984), demonstrating that the nuclease activities of the enzyme are critical for the viability of the cell. In *E. coli*, Pol I plays an important role in correcting replication errors introduced by Pol III (Tago *et al.*, 2005). Moreover, Pol I in *E. coli* might compete with TLS polymerases for the access to ssDNA gaps on the lagging-strand (Maul, Sanders, *et al.*, 2007).

Therefore, next we questioned the role of TLS polymerases in cells lacking Pol I functions in *P. putida* cells. For that, we first constructed strains deficient in different Pol I functions: PaWPolA1 strain lacking the Klenow domain, but with retained 5′-nuclease activity, and PaWPolAdel and PaWPolAdel2 mutants, deficient in all domains (null allele of *polA*), and tested the mutants for the ability to grow in rich medium. The growth of PaWPolA1 strain with the remained 5′ nuclease function was not impaired on Luria-Bertani (LB) medium, suggesting that like in *E. coli*, the survival of the *polA*-deficient bacteria can be rescued by the presence of the nuclease function of the polymerase (Joyce and Grindley, 1984). In addition, similarly to *E. coli*, viability of *P. putida* strains deficient in all Pol I functions (PaWPolAdel and PaWPolAdel2 strains) was impaired in rich medium (Fig. 1A; Ref. II) and associated with the formation of filaments (Fig. 2C; Ref. II). However, to our surprise, during the 2–3 rounds of inoculation of stationary-phase cells of the Pol I-deficient strains into fresh rich medium, all Pol I-null mutants gained suppressor mutations that allowed bacteria to grow well on LB plates. These mutants with enhanced growth abilities in rich medium took over the population, restored the plating efficiency on LB plates and reduced filamentation of cells in fresh LB cultures. This unexpected finding and instability of Pol I-deficient bacteria in rich medium led us to analyze the role of Pol I and TLS polymerases in spontaneous mutagenesis in the PaWPolAdel2 strain lacking all Pol I functions, which was adapted to growth in LB (next in the thesis is indicated as PaWPolA for clarity).

The deletion of Pol I in *P. putida* resulted in the moderate increase (about 8-fold) in the spontaneous mutation frequency in comparison to the wild-type strain (PaW85), and to a drastic change in the spectra of mutations detected in the *rpoB* gene, demonstrating the importance of Pol I in fidelity of DNA replication (Table 4; Ref. II). The most dramatic change was observed in the number of deletions in the Pol I-deficient bacteria in comparison to the wild-type cells. With only one deletion found out of 167 mutants analyzed in the wild-type bacteria ($6.97 \times 10^{-9} \times 1/167 = 0.041 \times 10^{-9}$), the frequency of deletions in the Pol I-deficient strain increased almost 200 times ($59.21 \times 10^{-9} \times 15/92 = 9.65 \times 10^{-9}$). What is more interesting, all deletions detected in the Pol I-deficient Rif^r mutants started at the position 1611 and encompassed 6, 9 or 12 nucleotides (Table 4; Ref. II). Moreover, the analysis of base substitutions demonstrated drastic changes in the distribution of spontaneous mutations in the *rpoB* gene in Pol I-deficient background (Tables 4 and 5; Ref. II). For example, in comparison

with the wild-type strain, in the absence of Pol I the number of A→G transitions at the position 1553 was almost 40-times higher (0.58×10^{-9} in the wild-type strain and 22.53×10^{-9} in the Pol I-deficient bacteria), representing a mutational hotspot in bacteria lacking Pol I functions. The deletion of Pol I also resulted in the appearance of some additional base substitutions that were previously not detected in the spectrum of wild-type bacteria: for example, T→C transitions at the 1547 and 1613 positions, and A→T transversions at the 1562 site of the *rpoB* gene. As such, these results demonstrate that in the absence of Pol I in *P. putida,* the increased frequency of mutations is associated with the increased number of replication errors, both deletions and base substitutions at certain positions in the *rpoB* gene.

Changes observed in the spectra of Pol I-deficient strain could be attributed to unrepaired errors introduced during replication by Pol III or by alternative DNA polymerases involved in re-filling of ssDNA gaps in the absence of Pol I. To test this possibility, we analyzed the mutation rates and spectra in *P. putida* Pol I-deficient bacteria lacking additionally Pol II (PaWPolAPolB), Pol IV (PaWPolADinB) or ImuC (PaWPolADnaE2). As shown in Table 4 (Ref. II), the spontaneous mutation frequencies in strains deficient in either Pol II, Pol IV or ImuC were comparable to the frequency observed in the parental strain carrying deletion in Pol I function, suggesting that none of the TLS polymerases alone could be responsible for the increased number of mutations in Pol I-deficient bacteria. As such, we can assume that increased spontaneous mutation frequency in the Pol I-deficient bacteria could be associated with the actions of Pol III (or interplay of specialized DNA polymerases), but not by specific TLS polymerase alone. Nevertheless, the analysis of the mutational spectra in the Pol I-deficient bacteria lacking Pol II and Pol IV functions revealed that both Pol II and Pol IV might get access to DNA in the absence of Pol I (Table 5; Ref. II). For example, the A→T transversions at the 1562 site of the *rpoB* gene, an important hot spot in the Pol I-deficient strain (5.15×10^{-9}), were absent or significantly underrepresented in *P. putida* Pol I-deficient bacteria lacking Pol IV and Pol II (1.25×10^{-9}), respectively (Table 4; Ref. II). At the same time, this decrease was accompanied by a significant elevation in A→G transitions at the same site, from 2.57×10^{-9} in Pol I-deficient bacteria to 20.62×10^{-9} in PaWPolAPolB and to 16.08×10^{-9} in PaWPolADinB. Therefore, in the Pol I-deficient background Pol IV and Pol II might be responsible for these changes, either suppressing or promoting specific types of mutation. Notably, in Pol IV- and Pol II-deficient strains in the wild-type background this A→G mutation at the 1562 site was on the contrary dependent on the presence of TLS polymerases, suggesting their different contribution to the appearance of mutations in Pol I-proficient and -deficient backgrounds. The opposite effect was also observed on the occurrence of A→G at the 1553 position: the presence of Pol IV and Pol II suppressed this type of mutation in the wild-type background. However, in Pol I-deficient bacteria the lack of Pol IV and Pol II led to the decrease in the frequency of this mutation from 22.53×10^{-9} in Pol I-deficient bacteria to 11.35×10^{-9} ($P < 0.005$) in PaWPolADinB strain and to

6.9×10^{-9} in PaWPolAPolB ($P < 10^{-4}$). Taken together, this data suggests that both Pol II and Pol IV could get access to DNA in the absence of Pol I, and the sequence context might influence the mutagenic activity of TLS polymerases at the particular sites in the *rpoB* gene. Moreover, opposite effects on the occurrences of mutations observed in Pol I-proficient background, suggests that aces of Pol IV and Pol II to DNA or their mutability in cells might be modulated by Pol I.

Interestingly, but no significant effect of the ImuC deficiency on the spectrum of mutations was observed in Pol I-deficient bacteria (Table 5; Ref. II), suggesting that ImuC activity might be either not mutagenic to the cell, being either outcompeted by other polymerases or simply not involved in DNA replication in the absence of exogenous DNA damage. On the other hand, such nonmutagenic activity of ImuC in the absence of exogenous damage is in accordance with a recent study in *C. crescentus*, where it was demonstrated that overexpression of the *imuABC* operon at the level which is common for the SOS induction had no effect on the spontaneous mutagenesis (Alves *et al.*, 2017).

Some earlier studies with *E. coli* using the histidine region, *lacI* or endogenous *tonB* as reporter genes have reported that mutations in the *polA*-encoded Pol I resulted in the appearance of deletions and minus frameshift mutations (Savić and Romac, 1982; Fix *et al.*, 1987; Agemizu *et al.*, 1999). It was demonstrated that mutation in the Klenow domain led to a marked increase in deletions and minus frameshift mutations, whereas mutation in the 5′→3′ exonuclease led to an increase in duplications and plus frameshifts (Agemizu *et al.*, 1999; Nagata *et al.*, 2002). At the same time, no significant effect of the *polA*-deficiency on the occurrence of spontaneous base substitutions was observed by using the chromosomal *tonB* reporter system (Agemizu *et al.*, 1999; Nagata *et al.*, 2002). In addition, this group also demonstrated that in *E. coli,* specialized DNA polymerases did not contribute to spontaneous mutagenesis in bacteria lacking Pol I functions (Tago *et al.*, 2005). Despite chronical induction of the SOS response in Pol I-deficient strain (Nagata *et al.*, 2003), no effect of TLS polymerases on the rate or spectra of mutations in the chromosomal *tonB* gene was observed, suggesting that Pol III solely might be responsible for mutations observed in the Pol I-deficient strain (Tago *et al.*, 2005). The analysis of spontaneous mutagenesis using the *rpoB*/Rif^r system in *E. coli* strain deficient in Pol I proofreading function but retaining polymerase function revealed the 2-fold increase in level of spontaneous mutations, which was associated with the increase in base substitutions; however, deletions were not detected (Makiela-Dzbenska *et al.*, 2011). Because of the essential function of RNAP in cells, it is impossible to detect any frameshift mutations by employing the *rpoB*/Rif^r reporter assay. As such, the observed signatures of Pol I observed by using one system cannot be detected by other reporter system.

Our results demonstrated that in *P. putida,* Pol I-deficiency resulted in almost 9-fold increase in the spontaneous mutation frequency in the *rpoB* gene, demonstrating the importance of this polymerase in maintaining of replication

fidelity. There was a drastic increase in both transversion and transitions rates, however almost 16% of the detected mutations were deletions (Table 4; Ref. II). In addition, we observed the possible involvement of Pol II and Pol IV in replication of chromosomal DNA in the absence of Pol I, and what is more interesting, the effect on the occurrences of some mutations was different if to compare with the Pol I-proficient background. None of the TLS polymerases alone contributes to the increased mutagenesis in Pol I-deficient bacteria, suggesting that Pol III might be responsible for the replication errors. These results suggest that in the absence of one polymerase the other polymerase(s) can promote or suppress mutation depending on the sequence context, resulting in the overall balance in mutagenic load, indicating constant interplay between polymerases inside the cell.

3.2.3 The involvement of ImuC in UV-induced mutagenesis

All living organisms are constantly exposed to a wide range of external (chemical and physical) factors that can alter the integrity of DNA molecule. Ultraviolet (UV) exposure induces in DNA multiple photoproducts, with cyclobutane pyrimidine-pyrimidine dimers and pyrimidine (6–4) pyrimidone adducts being most frequent and toxic to cell by inhibiting transcription and replication (Barak *et al.*, 1995). Removal and repair of the UV-induced lesions occurs mostly through NER pathway, mediated by UvrA, UvrB, UvrC and UvrD proteins (Van Houten, 1990). After DNA damage excision and removal, 12–13 nt gap is formed, which is further filled in by Pol I, and the newly synthesized end is further resealed by DNA ligase into continuous DNA strand (Truglio *et al.*, 2006; Kisker *et al.*, 2013). Therefore, in the absence of Pol I, the lesions would be excised, but the resulting ssDNA gaps would be filled in by other DNA polymerases present in cell.

Unrepaired damaged sites can arrest the progression of replication fork, leading to re-priming downstream the lesion and generation of ssDNA gaps. Subsequently these gaps can be repaired via recombinational repair or filled by TLS polymerases (Naiman *et al.*, 2016). In addition, TLS polymerases can bypass UV-induced bulky adducts by switching with replicative polymerase at the damaged site, rescuing arrested replication forks directly (Courcelle *et al.*, 2005; Lehmann and Fuchs, 2006). However, tolerance of UV-induced damage via TLS can also lead to mutations. Both photoproducts covalently join adjacent pyrimidines, and insertion of incorrect bases by TLS polymerase leads to the appearance of C→T substitutions at dipyrimidine sites and CC→TT tandem changes, a signature of so-called “UV-specific” mutations (Brash, 2015). In *E. coli,* the bypass of UV-induced cyclobutane pyrimidine dimers depends entirely on the activity of mutagenic Pol V (Szekeres *et al.*, 1996; Tang *et al.*, 2000; Wrzesiński *et al.*, 2005). With the absence of UmuDC-encoded Pol V in many bacteria species carrying instead the “mutagenesis cassette”, it was suggested that products of this operon might be involved in UV-damage

tolerance in these organisms (Erill *et al.*, 2006). So far, the role of ImuC in UV-induced mutagenesis have been controversial. In *M. tuberculosis* (Warner *et al.*, 2010), *C. crescentus* (Galhardo *et al.*, 2005), as well in *P. aeruginosa* (Sanders *et al.*, 2006) the UV-induced mutagenesis has been demonstrated to depend upon ImuC. On the contrary, in *P. putida* wild-type background the ImuC was shown to have antimutator effect (Koorits *et al.*, 2007).

Consequently, we prompted to analyze the potential role of ImuC in *P. putida* in the tolerance of UV-induced lesions in bacteria lacking Pol I functions. In *P. putida,* the Pol I-deficiency increased remarkably the sensitivity of bacteria to UV irradiation (Fig. 3; Ref. II). At the same time, ImuC, as well as Pol IV and Pol II (or three of them together) were not required for the UV-induced damage tolerance (data not shown). Surprisingly, in both *P. putida* wild-type and in bacteria lacking Pol I functions, UV irradiation had no effect on the frequencies of mutations. The estimated frequencies of UV-induced Rif^r mutations were comparable to those observed in non-treated cells (Tables 4 and 6; Ref. II), demonstrating the absence of UV-induced mutagenesis in *P. putida* at the conditions tested. Yet, analysis of mutational spectra in irradiated bacteria revealed significant differences in comparison with non-irradiated cells (Tables 5 and 6; Ref. II). For example, in irradiated wild-type bacteria, we observed a new hot spot in the C→T transitions at the position 1706 that was previously not detected in non-treated cells. In addition, there was an increase in the A→G transitions at the position 1553 of the *rpoB* gene (increase from 0.58×10^{-9} in non-treated to 1.74×10^{-9} in irradiated cells). Surprisingly, the increase in the frequency of this particular mutation was accompanied by a concurrent decrease in the same mutation type at the position 1592 (decline from 0.75×10^{-9} in non-treated cells to 0.17×10^{-9} in UV-treated bacteria). In the spectrum of UV-irradiated Pol I-deficient bacteria, there was a 4-fold decrease in the A→G transitions at the positions 1553, which was a hotspot in non-treated Pol I-deficient cells. The decrease at one position was accompanied by up to 10-fold increase in the same mutation type at the position 1562 (from 2.57×10^{-9} to 27.77×10^{-9}). In addition, the frequency of A→T transversions was increased at the position 1992. Surprisingly, the number of deletions observed in the UV-irradiated Pol I-deficient bacteria was almost 5-times lower than in non-treated cells (Tables 4 and 6; Ref II).

In attempt to gain the insight into the possible involvement of ImuC in the replication past UV-induced damage, we analyzed the number and spectra of mutations in irradiated *P. putida* strain lacking both Pol I and ImuC functions. The analysis revealed that there was no significant effect of ImuC deficiency on the UV-induced mutation frequencies in the Pol I-deficient background, suggesting that in *P. putida* ImuC does not contribute to the UV-induced mutagenesis. However, UV-irradiation led to a significant change in the mutation spectra (Tables 5 and 6; Ref. II). First of all, there was a striking decrease in the number of the A→G transitions at the position 1562 (up to 9 times) and the A→T transversions at the positions 1553 and 1592 of the *rpoB* gene in the irradiated PaWPolADnaE2 strain, compared to the parental PaWPolA. These

results suggest that in Pol I-deficient bacteria these mutations could be associated with the erroneous activity of ImuC. If we look at the sequence specificity of the ImuC-induced mutations, then in both cases the A→T transversions appeared when the adjacent 5′ nucleotide was C (5′-CCAG-3′ and 5′-CCAC-3′, respectively). On the other hand, the absence of ImuC also led to a significant increase in the A→G transitions at the 1553 site, and the appearance of the T→C transitions at the 1547 and 1549 positions (sites with adjacent pyrimidines 5′-CT-3′ and 5′-TCC-3′, respectively), suggesting also antimutagenic activity of this TLS polymerase within some genomic context.

The specificity of overall types of mutations observed in the UV-irradiated spectra of the wild-type strain resembled the spectrum of non-treated bacteria (Table 1), suggesting efficient repair of UV-induced damage. Yet, in irradiated Pol I-deficient strain there was a slight increase in the overall number of A:T→G:C transitions and A:T→T:A transversions. On the contrary, in irradiated bacteria lacking both Pol I and ImuC (PaWPolADnaE2 strain) there was a drastic decrease in A:T→T:A mutations, demonstrating that in UV-treated Pol I-deficient bacteria ImuC might be responsible for the occurrence of A:T→T:A substitutions (Table 1). Moreover, A:T→G:C transitions in UV-treated PaWPolADnaE2 bacteria represented almost 83% of all detected mutations (while in UV-irradiated Pol I-deficient bacteria only 59%), and such increase was associated with the appearance of T→C substitutions in the spectra of mutations. Despite the small number of mutants analyzed, there is a tendency to assume that in irradiated Pol I-deficient bacteria, ImuC might partly suppress the appearance of these mutations. Thus, we can speculate that in UV-treated Pol I-deficient bacteria ImuC might not only promote the appearance of A:T→T:A transversions, but also suppress some mutations, especially those produced at the adjacent pyrimidine sites, probably by mediating accurate bypass past some of the UV-induced damage.

TLS polymerases are involved in tolerance and mutagenesis of UV-induced damage in several studied bacteria, *e.g.*, in *E. coli, M. tuberculosis* and *C. crescentus*. In *E. coli,* TLS polymerase-deficient strains exhibit modestly increased UV sensitivity, and UV-irradiation usually leads to a robust increase in the frequency of Rif^r mutants with Pol V responsible for C→T transitions and other UV-induced mutations (Courcelle *et al.*, 2005; Janel-Bintz *et al.*, 2017). The G:C→C:G changes in the *rpoB* gene of the UV-irradiated *C. crescentus* were identified as the UV-dependent mutagenic signature of the TLS activity performed by ImuABC (Galhardo *et al.*, 2005). The *imuC*-dependent signature of the UV-induced mutations in *M. tuberculosis* was different, representing mostly double CC→TT transitions (Boshoff *et al.*, 2003). However, in irradiated *P. putida* cells*,* both these mutation types were never detected. Despite the use of different genetic backgrounds, we still can assume that mutagenic processes and mutagenic signatures of ImuABC might be different in these bacteria species. This can reflect the intrinsic different preferences of polymerase during TLS or fundamental differences and repair abilities of the organisms. The absence of UV-induced mutagenesis in *P. putida* cells suggest that UV-lesions

are efficiently repaired. However, in Pol I-deficient bacteria, ImuC is responsible for the occurrence of A:T→T:A transversions (and also probably suppression of some of the A:T→G:C transitions). This clear shift in this mutation type undoubtedly revealed the specificity of ImuC in *P. putida*. Since no difference in the frequency of this mutation type was observed in the spectrum of irradiated wild-type bacteria, we can assume that ImuC might be under strict regulation in the wild-type background, or ImuC-mediated mutations might be efficiently repaired. As such, new studies are needed to understand these mechanisms in more detail.

Table 1. Frequencies of spontaneous and UV-induced mutations detected in the *rpoB* gene in *P. putida* wild-type cells (PaW85) and its polymerase-deficient derivatives PaWPolA (deficient in Pol I) and PaWPolADnaE2 (deficient in Pol I and ImuC).

	Spontaneous						**UV-induced**					
	PaW85		**PaW PolA**		**PaWPolA DnaE2**		**PaW85**		**PaW PolA**		**PaWPolA DnaE2**	
	N	***MF***	*N*	***MF***	*N*	***MF***	*N*	***MF***	*N*	***MF***	*N*	***MF***
Transitions												
G:C→A:T	30	**1.3**	9	**5.8**	7	**6.6**	23	**2.0**	6	**4.6**	2	**2.1**
A:T→G:C	95	**4.0**	43	**27.7**	34	**32.2**	44	**3.8**	46	**34.9**	40	**41.0**
Transversions												
A:T→T:A	25	**1.0**	22	**14.2**	13	**12.3**	11	**1.0**	23	**17.5**	1	**1.0**
A:T→C:G	2	**0.1**					3	**0.3**				
G:C→T:A	12	**0.5**	2	**1.3**								
G:C→C:G	2	**0.1**										
Del	1	0.04	15	**9.7**	10	**9.5**			2	**1.5**	5	**5.1**
Ins			1	0.6								
All:	167	**7.0**	**92**	**59.2**	**64**	**60.6**	81	**7**	77	**58.5**	48	**49.2**

Rif^r mutation frequencies per 10^9 cells are indicated as *MF*, *N* indicates the overall number of mutations detected (Data is taken from Table 4 and Table 6, Ref. II)

3.3 Alkylation damage repair and tolerance in *Pseudomonas* species (Reference III)

Alkylating agents are constantly formed during normal metabolism in cell (as was briefly discussed in Chapter 1.2.2.3 of this thesis), but are also found everywhere in the environment, including air, water, food and pollutants, and are widely used in cancer therapy (Hecht, 1999; Hurley, 2002). Almost all atoms in DNA can be susceptible to alkylation damage (Gates, 2009). The most frequent methylation products formed in dsDNA are N^7-methylguanine (N^7meG, accounting for 60–80% of the total lesions) and N^3-methyladenine (N^3meA, 10–20%) (Fu *et al.*, 2012). N^7meG itself is relatively harmless, since methyl group does not block replication or modify the coding specificity of the nucleotide. However, this lesion is prone to hydrolysis that can lead to the formation of apurinic/apyrimidinic (AP) sites (Tudek *et al.*, 1992; Tudek, 2003). Formed at the minor groove of the DNA at the N^3 of purines, both N^3meA and N^3meG lesions are highly toxic to cells, representing block to high-fidelity DNA polymerases (Wyatt and Pittman, 2006). Some agents also induce lesions in ssDNA, for example, N^1-methyladenine (N^1meA) and N^3-methylcytosine (N^3meC), which are induced by methyl methanesulfonate (MMS), inhibit base pairing and thus block replicative DNA polymerases (Wyatt and Pittman, 2006). *O*-alkylation adducts, O^6-methylguanine (O^6meG) and O^4-methylthymine (O^4meT) are highly mutagenic, and under some circumstances O^6meG can have cytotoxic effect (Lindahl, 1996; Fu *et al.*, 2012).

To deal with alkylation DNA damage cells have evolved multiple specific damage repair strategies, which mediate either direct repair of primary alkylation damage (methyltransferases and dioxygenases) (Fig. 14 A, B), or represent multistep repair mechanisms, like DNA-glycosylase initiated BER (Fig. 14). *O*-alkylation is repaired in a one-step reaction by methyltransferases: these enzymes directly transfer the methyl adducts from the oxygen onto its own nucleophilic cysteine residues of the active site (Fig. 14A). For example, in *E. coli* there are two enzymes responsible for the repair of mutagenic *O*-methyl adducts: DNA damage-inducible Ada and constitutively expressed Ogt (Sedgwick, 1983; Takahashi *et al.*, 1988; Sedgwick and Lindahl, 2002). Oxidative demethylases (in *E. coli* enzyme AlkB) repair cytotoxic N^1meA and N^3meC lesions by direct reversal of adducts via oxidation of the alkyl group (Fig. 14B) (Falnes *et al.*, 2002; Aas *et al.*, 2003). The repair of *N*-methyl adducts, including N^7meG, and toxic N^3meA and N^3meG, is mediated by BER (Fig. 14C). The repair is initiated by lesion-specific DNA glycosylases that recognize and hydrolyze the *N*-glycolysic bond of the damaged base, generating AP site, which is subsequently processed via BER pathway. *E. coli* possesses two DNA glycosylases: constitutively expressed DNA glycosylase I, or Tag, and damage-inducible DNA glycosylase II, AlkA. Tag only mediates the removal of N^3meA and N^3meG (Bjelland *et al.*, 1993), however, AlkA has a much wider lesion specificity, additionally removing N^7meG and miscoding *O*-alkylated pyrimidines, such as O^2-methylcytosine and O^2-methulthymine (McCarthy *et al.*,

1984; Krokan *et al.*, 1997). All these pathways represent error-free repair systems, playing important role in protection of cells from toxic and mutagenic consequences of methylation damage. In addition, NER, TLS, HR and NHEJ are involved in the repair and tolerance of either primary methylation damage or secondary DNA lesions, and imbalances in any of the pathways have a dramatic consequence on genome stability (Drabløs *et al.*, 2004; Fu *et al.*, 2012). TLS has shown to be critical for the tolerance of alkylation damage both in *E. coli* (Bjedov *et al.*, 2007), as well as eukaryotic cells (Fig. 14D) (Johnson *et al.*, 2007; Yoon *et al.*, 2017). For example, in *E. coli*, Pol IV contributes to the ability of bacteria to survive alkylation damage by mediating accurate bypass of alkylated DNA adducts *in vivo*, including MMS-induced N^3meA lesions (Bjedov *et al.*, 2007; Benson *et al.*, 2011; Scotland *et al.*, 2015). The second Y-family member, Pol V, does not influence the survival of *E. coli* in the presence of MMS; however, it is still involved in TLS across MMS-induced damage, as deficiency in Pol V results in the reduction of the MMS-induced mutations (Bjedov *et al.*, 2007). Therefore, next task of my work was to elucidate the role of TLS polymerases ImuC and Pol IV in *P. putida* and *P. aeruginosa* in response to alkylation damage.

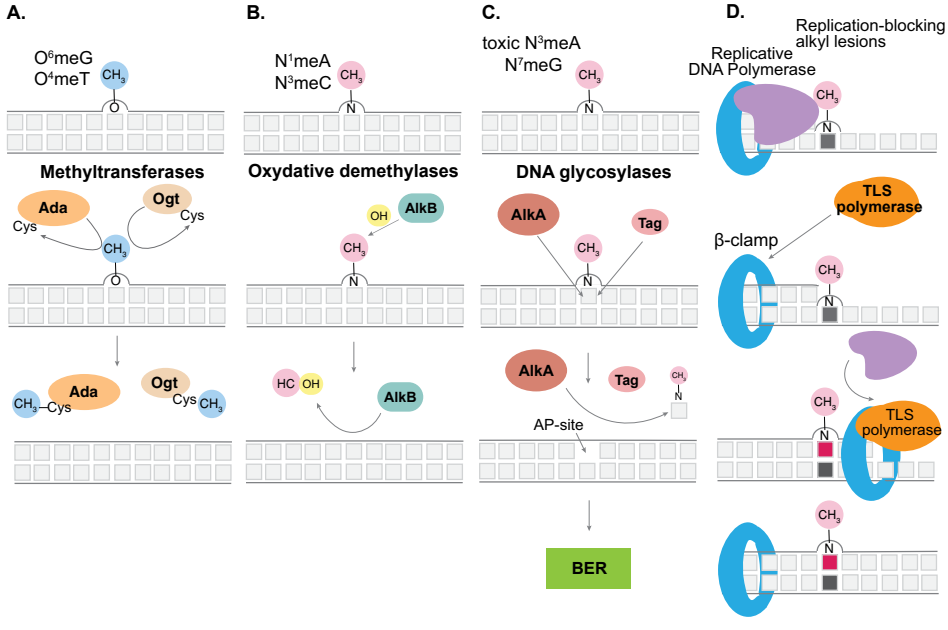

Figure 14. Alkylation damage repair (A–C) and tolerance (D). Direct *O*-alkyl damage repair by methyltransferases Ada and Ogt (A), and *N*-alkyl damage repair by oxidative demethylase AlkB (B) results in the reversal of DNA damage into a normal base. DNA glycosylases AlkA and Tag mediate the excision of the damaged base, generating toxic AP site, which is further repaired via BER pathway (C). TLS polymerases can rescue replicative polymerase stalled at the damaged site by mediating translesion DNA synthesis, or fill in lesion-containing gaps and, therefore, increase cell survival (D).

3.3.1 Differential role of TLS in alkylation damage tolerance in Pseudomonads

First, we wanted to elucidate whether TLS contributes to survival of Pseudomonads following exposure to alkylating agents. For that, we created a set of *P. aeruginosa* PAO1 (PAO1-L subline) and *P. putida* PaW85 polymerase-deficient derivatives and tested the contribution of TLS polymerases to survival in the presence of two alkylating agents: methyl methanesulfonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). MMS and MNNG generate a plethora of DNA lesions, but in dsDNA both primarily induce N^7meG and toxic N^3meA lesions. Differently from MMS, MNNG, in addition to *N*-alkyl lesions, generates also significant proportion of O^6meG (7% generated by MNNG and only 0.3% by MMS), however, MMS also produces toxic N^1meA and N^3meC lesions in ssDNA (Beranek, 1990).

To test the sensitivity of TLS-deficient strains to methylating agents, we spotted stationary-phase bacteria onto plates containing MMS or MNNG and scored colony-forming units (CFU) after 24 or 48h incubation period. We observed that the lack of Pol IV (Δ*dinB* strain) in *P. aeruginosa* resulted in the increased sensitivity to MMS (Fig. 1A; Ref. III), demonstrating the importance of Pol IV in the tolerance of MMS-induced damage. Unlike the deletion of the *dinB*, ImuC-deficient *P. aeruginosa* was as sensitive as the wild type. However, strain deficient in both Pol IV and ImuC (Δ*imuCdinB*) was significantly more sensitive to MMS than *P. aeruginosa* strain lacking Pol IV only, suggesting that ImuC might be also involved in the tolerance of methyl damage. At the same time, in *P. putida* TLS polymerases had no effect on the survival on MMS-containing plates: sensitivity of cells lacking Pol IV and ImuC, as well bacteria lacking all TLS polymerases, including Pol II (data not shown), was comparable to the sensitivity of the wild-type bacteria (Fig. 1C; Ref. III). Therefore, these results indicate that MMS-induced damage in *P. putida* and *P. aeruginosa* could be tolerated differently, with TLS playing important role in survival in *P. aeruginosa,* while in *P. putida* all MMS-induced damage could be efficiently repaired or tolerated by different mechanisms.

However, in contrast to MMS, in the presence of MNNG, sensitivity of *P. putida* strain lacking Pol IV was slightly increased (Fig. 1D; Ref. III). Moreover, *P. putida* and *P. aeruginosa* (Fig. 1B; Ref. III) Δ*imuCdinB* double deficient strains were significantly more sensitive to this agent. As MNNG, differently from MMS, induces also a significant proportion of oxygen adducts, we can assume that the increased sensitivity of *P. putida* strain lacking TLS could pinpoint at the potential involvement of these TLS polymerases in the bypass or repair of *O*-alkylation damage. On the other hand, global transcriptional analysis of MMS- and MNNG-treated *Saccharomyces cerevisiae* cells has revealed that these chemicals, despite similarity in the pattern of alkylation lesions, induce remarkably different and unique subset of genes (Jelinsky *et al.*, 2000). Therefore, the difference in the expression of repair/tolerance pathways or in the repair efficiencies of distinct types of methylation damage could probably explain

the differential role of TLS detected in *P. putida* cells upon given experiment conditions.

In *E. coli,* genes encoding proteins involved in alkylation DNA repair constitute adaptive response (Sedgwick and Lindahl, 2002). This set is comprised of *ada, aid, alkA*, and *alkB* genes. However, the adaptive response is induced not only in response to exogenous alkylation damage, but also when cells enter stationary phase, to protect bacteria against endogenously formed alkylators and spontaneous DNA damage (Taverna and Sedgwick, 1996; Sedgwick, 1997; Landini and Volkert, 2000). Interestingly, in yeast, the transcriptional profiles in response to methylation-induced damage greatly overlaps with the profile of stationary-phase cells (Fry *et al.*, 2005). Therefore, stationary-phase cells could be more ready to counteract alkylation damage than exponentially growing cells. With regard to this and to the fact, that during the incubation of *P. putida* on plates containing MMS, colonies appeared only 48 hours after spotting, we tested the survival of *P. putida* TLS-deficient strains following treatment with high concentration of MMS also during exponentially growth of bacteria. It appeared that the survival of Δ*dinB* strain lacking Pol IV and especially of Δ*imuCdinB* double mutant upon MMS-treatment was significantly lower than the survival of the wild-type strain (Fig. 2; Ref. III), revealing the importance of TLS for the survival of MMS-induced damage in exponentially growing cells.

Taken together, our results demonstrate that in *P. putida* and *P. aeruginosa* both Pol IV and ImuC might be involved in the tolerance of alkylation damage. However, differences in MMS sensitivities observed in two representatives of *Pseudomonas* suggest that the repair efficiencies or specificities of these organisms could be different, resulting in distinct importance of TLS for the survival of MMS-induced damage. The supreme abilities of *P. putida* to tolerate MMS-induced damage could be attributed to the existence of additional copies of enzymes responsible for alkylation damage repair. In particular, this organism, in addition to conserved *alkA* and *tag* genes, encoding for DNA glycosylases II and I, respectively, has additional copies of genes encoding for N^3meA DNA glycosylases (Mielecki *et al.*, 2013). Moreover, in contrast to *E. coli*, the expression of *alkB* in *P. putida* is constitutive (Mielecki *et al.*, 2013). Therefore, *P. putida* not only efficiently tolerates UV-induced damage (Ref. II), but also MMS-mediated alkylation lesions, with TLS playing critical role only during active growth of bacteria.

3.3.2 Both Pol IV and ImuC facilitate survival of DNA glycosylase-deficient bacteria upon the MMS- and MNNG treatment

To specify the role of Pol IV and ImuC in DNA alkylation-damage tolerance, we have constructed strains lacking additionally genes involved in alkylated DNA repair. Based on the studies in *E. coli,* the majority of replication-blocking DNA lesions induced by MMS are removed in cells via BER pathway, which is initiated by lesion-specific DNA glycosylases AlkA and Tag. Both glycosylases

excise N^3meA and N^3meG; however, AlkA is also responsible for the removal of N^7meG and alkylated *O*-pyrimidines (Bjelland *et al.*, 1993; Bjelland and Seeberg, 1996). Recent study in *P. putida* has revealed, that DNA glycosylase AlkA might also be involved in the removal of toxic N^1meA and N^3meC lesions induced by MMS in ssDNA (Mielecki *et al.*, 2013). Consequently, the deletion of *alkA* and *tag* will lead to the accumulation of *N*-alkyl damage, which would allow specifying role for Pol IV and ImuC in alkylation lesion bypass. Therefore, we constructed a set of TLS-deficient strains also deficient in DNA glycosylase II (Δ*alkA*) and two DNA glycosylase (Δ*alkAtag*) functions in *P. putida,* and Δ*alkA*-deficient derivatives of TLS-lacking *P. aeruginosa* strains, and tested the susceptibility of bacteria to methylating agents.

First, we observed the dramatic increase in MMS- and MNNG-sensitivity of strains deficient in DNA-glycosylase functions in both *P. putida* and *P. aeruginosa* (Fig. 3; Ref III), revealing and supporting the critical role of the glycosylase-initiated BER in protection of cells against alkylation damage. If the effect of TLS-deficiency on MMS sensitivity in the wild-type *P. putida* and *P. aeruginosa* was different (Fig. 1; Ref. III), then in Δ*alkA*-deficient strains we were surprised to observe quite opposite picture, as both ImuC and Pol IV appeared to be essential for the survival of both species (Fig. 4 and 5; Ref. III). Moreover, the survival pattern of *P. putida* and *P. aeruginosa* Δ*alkA*-deficient strains lacking Pol IV and ImuC was the same: MMS-sensitivity of the Δ*imuCalkA* strains was similar to that of Pol IV-deficient bacteria (Fig. 4A and 5A; Ref. III) with Δ*imuCdinBCalkA* polymerase double mutant displaying additive increase in sensitivity. Interestingly, in the response to MNNG, both *P. putida* and *P. aeruginosa alkA*-deficient bacteria lacking additionally ImuC were more sensitive than those lacking Pol IV. Besides, sensitivity of the strains deficient in both TLS polymerases was comparable to the sensitivity of Δ*imuCalkA* strains (Fig. 4B and 5B; Ref. III), demonstrating that ImuC might be more important for the survival of MNNG-induced damage.

The increased sensitivity of ImuC- and Pol IV-deficient bacteria in AlkA- (Fig. 4 and 5; Ref. III) and AlkATag-deficient background (Fig. 4C and 4D, Ref. III) suggests that ImuC and Pol IV are involved in replication past lesions normally repaired by DNA glycosylase-mediated repair. In addition, both TLS polymerases might be involved in the bypass past different MMS-induced lesions, since sensitivity of Δ*imuCdinB* double mutants was significantly higher in comparison with the sensitivity of single mutants. If in *E. coli* AlkA and Tag-deficient bacteria are sensitive to alkylation damage because of the accumulation of unrepaired toxic N^3meA and N^3meG lesions (Bjelland *et al.*, 1993), then in *P. putida* this list could be broader. Due to the wider specificity of AlkA enzyme, the sensitivity of bacteria, deprived of functional glycosylase-mediated repair, can be also attributed to toxic N^1meA and N^3meC lesions (Mielecki *et al.*, 2013). Therefore, we can assume that ImuC and Pol IV in *P. putida* could mediate bypass past MMS-induced N^3meA, N^3meG, N^1meA and N^3meC lesions. Our observations on the increased MNNG-sensitivity of the AlkA-deficient bacteria lacking ImuC but not Pol IV (Fig. 4B and 5B, Ref. III)

suggest that ImuC might be involved in the bypass of *O*-methylated bases. Both MMS and MNNG generate almost similar amount of *N*-alkyl damage (Beranek, 1990); however, MNNG-induced O^6meG lesions are suspected to be not only mutagenic but also lethal in *E. coli* cells (Nowosielska *et al.*, 2006). Therefore, our data unexpectedly revealed the importance of ImuC in protection against MNNG-induced damage in Pseudomonads deficient in AlkA-mediated repair.

Nevertheless, the toxicity from methylating agents results not only from the generation of methylation adducts but also from lethal intermediates, namely AP sites, that appear as a result of spontaneous hydrolysis of unstable *N*-methylpurines (Larson *et al.*, 1985; Loeb and Preston, 1986). Depurination of predominant methylation N^7meG lesion is relatively slow (the half-life is app. 150h) (Gates *et al.*, 2004; Gates, 2009), and in the context of our experiments theoretically does not significantly contribute to the appearance of AP sites. On the other hand, N^3meA damage is in times less stable, with half-life ranging from 4 to 24h, being 40-fold shorter in ssDNA (Fronza and Gold, 2004; Gates, 2009; Shrivastav *et al.*, 2010). Consequently, accumulating unrepaired N^3meA lesions in glycosylase-deficient background not only directly but also through replication-blocking depurination intermediates could contribute to lethality of bacteria, especially those lacking TLS. Studies in *E. coli* suggest that both Pol IV and Pol V are able to insert nucleotide opposite AP sites *in vitro.* However, *in vivo,* bypass of the AP sites mostly depends on Pol V (Tang *et al.*, 2000; Maor-Shoshani *et al.*, 2003; Kroeger *et al.*, 2004; Weerasooriya *et al.*, 2014). Accordingly, in the absence of TLS, replicative polymerase stalling not only at alkylation lesions but also at AP sites can lead to the replication fork collapse, subsequent double-strand breaks, resulting in cell death (Fronza and Gold, 2004; Fu *et al.*, 2012). As such, we cannot exclude the possibility that also in Pseudomonads, in addition to methylation adducts, Pol IV and ImuC might be involved in the bypass of AP sites. However, further studies are needed to examine this possibility.

3.3.3 ImuC in responsible for MMS-induced mutagenesis in both *P. putida* and *P. aeruginosa*

The above-discussed results indicated that both ImuC and Pol IV are involved in the tolerance of methylation damage in Pseudomonads. However, is this bypass error-free or error-prone? In *E. coli*, TLS past MMS-induced damage, mediated by Pol V, is highly mutagenic (Grzesiuk and Janion, 1994; Nieminuszczy *et al.*, 2006; Sikora *et al.*, 2010), but Pol IV, in contrast, mediates accurate bypass (Bjedov *et al.*, 2007). Therefore, next task of the study was to elucidate whether TLS polymerases contribute to mutability of MMS in *Pseudomonas* species. For that, we incubated bacteria with low concentrations of MMS overnight in a liquid medium, to allow mutations to accumulate, and examined the frequency of mutations by scoring Rif^r mutants (Fig. 7A and 8; Ref. III). Since the growth of *P. putida* TLS-deficient Δ*alkAtag* strains at present

concentration was inhibited, the same experiment for Δ*alkAtag* bacteria was also repeated with lower doses of MMS (0.05mM) (Fig. 7B; Ref. III).

Our results demonstrated that in both *P. putida* and *P. aeruginosa,* the MMS-mutagenicity strongly depends on the presence of ImuC in cells (Fig. 7 and 8; Ref. III). In all tested backgrounds, frequencies of MMS-induced mutations were significantly lower in strains lacking ImuC if compared to the corresponding ImuC-proficient bacteria. On the contrary, in the Pol IV-deficient strains the frequency of the MMS-induced mutations was significantly higher. As such, this data indicated that also in *Pseudomonas* species Pol IV might mediate accurate bypass past MMS-induced damage, pointing to its evolutionary importance across different organisms (Bjedov *et al.*, 2007). In addition, the mutator phenotype observed in the Pol IV-deficient *P. putida* strains was dependent only on the ImuC function, as the mutant frequencies measured in the Δ*imuCdinB* strains were similar to those detected in bacteria lacking ImuC only. For example, the mutation frequencies in Δ*imuCalkA* and Δ*imuCalkAtag* did not differ significantly from the mutation frequencies observed in the corresponding Δ*imuCdinBalkA* and Δ*imuCalkAtag* strains, respectively. Taken together, our results demonstrated that in Pseudomonads ImuC and Pol IV have antagonistic effect on the appearance of MMS-induced mutations.

Studies in *E. coli* suggest that the presence of Pol IV in cells minimizes the mutagenic properties of Pol V, associated with error-prone bypass of AP sites (Bjedov *et al.*, 2007; Scotland *et al.*, 2015). This can be explained by the direct competition of Pol IV with Pol V for the access to the lesion and accurate Pol IV-mediated lesion bypass, or, as it was recently demonstrated, by indirect properties of Pol IV. Upon SOS-response induction, both Pol IV and RecA were shown to inhibit and slow down replication fork progression, allowing this way more time for DNA repair (Tan *et al.*, 2015). Hence, we can speculate that the same mechanism might be also translated onto *Pseudomonas* species, where either direct or indirect function of Pol IV could restrict the ImuC-mediated mutagenicity in the presence of alkylation damage and consequently affect overall genome integrity.

3.3.4 Incubation temperature affects DNA alkylation damage repair and tolerance in *P. aeruginosa*

The most unexpected results of our study were obtained when the growth temperature of Pseudomonads was shifted from their optimum. As already discussed above, the deletion of the *dinB* gene in *P. aeruginosa* led to the increased MMS sensitivity (Fig. 1A and 9A; Ref. III). These experiments were performed at 37 °C, the optimal growth temperature for *P. aeruginosa* (Tsuji *et al.*, 1982). However, when the same strains were incubated at 30 °C, then the increased MMS sensitivity appeared also in the absence of ImuC (Fig. 9A; Ref. III). Even more severe effect of the temperature on survival of TLS-deficient strains was observed in *P. aeruginosa* in the AlkA-deficient background. Namely, the

P. aeruginosa Δ*imuCalkA* strain was significantly more sensitive to MMS than strain deficient in Pol IV (Δ*dinBalkA*) (Fig. 9B; Ref. III), demonstrating that at lower temperature ImuC could be more important for the survival of bacteria than Pol IV. Concurrently, the growth of *P. aeruginosa* at lower temperature was significantly slower, as colonies on MMS-containing medium appeared on the second day after spotting. Consequently, to eliminate the effect of prolonged incubation, as the proportion of AP sites due to the spontaneous hydrolysis of accumulating *N*-methylpurines could significantly increase in time, we performed also killing experiment in liquid medium (Fig. 10, Ref. III). For that, exponentially growing cells were treated with MMS, washed and immediately spotted in parallel onto LB plates. The plates were thereafter incubated at different temperatures. The quantification of survival by counting CFU revealed that lower temperature slightly affected the survival of MMS-treated AlkA-deficient bacteria (Fig. 10; $P = 0.033$; Ref. III). At the same time, the survival of MMS-treated Δ*imuCalkA* bacteria at 30 °C was almost 10-times lower compared to that incubated at 37 °C ($P \leq 0.0001$). Taken together, our data revealed that ImuC might be more critical for the protection of *P. aeruginosa* against MMS-induced cytotoxicity at the temperature below the growth optimum.

We hypothesised that the observed increase in MMS sensitivity of the ImuC-deficient bacteria at lower temperature could be associated either with insufficient repair of MMS-induced damage and subsequent inability of Pol IV to solely protect cells against the increased damage levels, or due to the differential regulation of Pol IV. Therefore, to understand the possible temperature effects, we measured the transcriptional activity of the *dinB* gene at different incubation temperatures in both wild-type (Fig. 11A; Ref. III) and its *alkA*-deficient derivative (Fig. 11B; Ref. III). To our surprise, the level of transcription from the *dinB* promoter was higher in the MMS-treated bacteria incubated at 30 °C (Fig. 11, Ref. III). Since the transcription of the *dinB* gene is induced in response to DNA damage, these results hinted to the elevated levels of MMS-induced damage in cells incubated at lower temperature. Thus, it was possible that alkylation damage repair and tolerance mechanisms in *P. aeruginosa* grown at 30 °C could not be as efficient as at the optimal growth temperature. Consequently, Pol IV might be unable to protect cells against all the damage that accumulate in cells. To test this, we introduced plasmid carrying the *dinB* gene under the P_{tac} promoter into *P. aeruginosa* strains. Introduction of additional copies of Pol IV indeed led to the restoration of MMS tolerance of Δ*imuC*- and even Δ*imuCdinB*-deficient bacteria to the wild-type level (Fig. S2; Ref. III). In addition, this experiment also demonstrated that ImuC and Pol IV might be involved in the bypass past similar (at least partly) MMS-induced lesions. Taken together, our data suggested that under lower growth temperature Pol IV is unable to protect *P. aeruginosa* against methylation damage either due to diminished efficiency of repair or tolerance pathways or due to different regulation of these processes. As a result, ImuC becomes more important, representing an essential backup mechanism to Pol IV for survival. However, how general could be these mechanisms in the other Pseudomonads?

3.3.5 In *P. putida* incubation temperature affects the functionality of ImuC in alkylation damage tolerance

The effect of the growth temperature on the MMS-sensitivity of *P. putida* TLS-deficient strains was even more intriguing. The optimal growth temperature for *P. putida* is 30 °C, and, as it was described above, both the deletion of *imuC* and *dinB* in *P. putida alkA*-deficient strain increased the sensitivity of bacteria at the same extent, and the Δ*imuCdinBalkA* strain was hypersensitive to the MMS-induced damage (Fig. 4A and 9C; Ref. III). However, when the same strains were incubated at higher temperature, *i.e.,* at 37 °C, the effect of the deletion of *imuC* became phenotypically undetectable: the MMS-sensitivity of the Δ*imuCalkA* strain was similar to that of parental Δ*alkA* strain (Fig. 9C; Ref. III). Still, the sensitivity of the Pol IV-deficient Δ*dinBalkA* mutant was increased, but it was identical to that observed in Δ*imuCdinBalkA* strain, demonstrating that the presence of Pol IV, but not ImuC was necessary for the survival of the AlkA-deficient bacteria. The absence of phenotypic effects of ImuC- deletion on the survival of MMS-treated *P. putida* cells at 37 °C inspired us to examine MMS-induced mutagenesis at this temperature. The MMS-induced mutagenesis in the *P. putida* Δ*alkAtag* strain was strongly dependent on the presence of ImuC in cells (Fig. 7; Ref. III). However, when the same experiment was repeated at 37 °C, it resulted in the total disappearance of ImuC-dependent mutator phenotype (Fig. 12; Ref. III). The MMS-induced Rif^r mutant frequency measured in the Δ*alkAtag* strain incubated at 37 °C was almost 6.5-times lower than that estimated at 30 °C. Furthermore, it was similar to that detected in the Δ*imuCalkAtag* strain. Thus, not only the ImuC-dependent MMS-tolerance but also mutator phenotype vanished at 37 °C, suggesting that at higher temperature DNA alkylation damage tolerance in *P. putida* might be ImuC-independent.

We also noticed that when *P. putida* AlkA-deficient strains were incubated at 37 °C, then the survival of bacteria in the presence of MMS was higher than at lower temperature (Fig. 9; Ref. III). Such increase in the MMS-tolerance at higher temperature could be associated with decreased MMS-toxicity, resulting, for example, from enhanced repair efficiency of MMS-induced damage or efflux of the chemical from the cell. Therefore, next we measured cellular response to MMS-induced damage in bacteria incubated at different temperatures. To facilitate this analysis, we constructed the *lexA2* gene fusion with the *lacZ* reporter gene and monitored its transcriptional activity at different growth temperatures. The *lexA2* gene is co-transcribed in *P. putida* with the *imuABC* genes, and since this regulon is DNA-damage inducible (Abella *et al.*, 2004), it represents an indirect measure of DNA damage in cell. We measured the *β*-galactosidase activities at two time points: one hour after MMS addition (Fig. 13A; Ref. III), and after the overnight incubation (Fig. 13B; Ref. III). After one hour of incubation in the presence of MMS, the induction of the *lexA2* gene was significantly higher in bacteria incubated at 37 °C both in the wild-type ($P \leq 0.0001$) and in the AlkA-deficient derivative ($P \leq 0.01$) (Fig. 13A; Ref. III). However, after the overnight incubation, all differences disappeared, and the *β*-galactosidase

activities were similar at both temperatures (Fig. 13B; Ref. III). Notably, the deletion of the *alkA* gene led to significant increase in the induction of *lexA2* gene at both temperatures, being almost two times higher than in the wild-type bacteria (Fig. 13B; $P \leq 0.0001$; Ref. III.), thereby confirming the higher amounts of unrepaired DNA damage in bacteria deficient in AlkA-mediated repair.

Taken together, our results demonstrate that when grown at 37 °C, the MMS-damage tolerance and MMS-induced mutagenesis are independent of ImuC functions in *P. putida*. In addition, concurrent increase in survival in the presence of MMS cannot be solely explained by the difference in the toxicity of MMS. On the other hand, slightly faster growth of bacteria at 37 °C (data not shown) leading to prompt increase in the expression of the *lexA2* (as well also other responses) observed in bacteria after one hour of incubation, could suggest that at this temperature bacteria could be faster ready to counteract MMS-induced damage, resulting in better survival. Still, we cannot exclude other temperature-dependent metabolic or physiological changes that could happen in bacteria at different incubation temperatures (Tachdjian and Kelly, 2006; Gadgil *et al.*, 2008). Therefore, further experiments are needed to explain the temperature effects of the TLS-deficiency on the tolerance of alkylation damage in *Pseudomonas* species.

3.3.6 Role of ImuA and ImuB in Pseudomonads

The important piece of the ImuABC TLS puzzle is the role of ImuA and ImuB in this complex. Controversial and opposite roles of ImuB and ImuC observed in *P. putida* in UV- and stationary phase mutagenesis (Koorits *et al.*, 2007) are in conflict with the data available in other bacteria. In *M. tuberculosis* and *C. crescentus* all products of the *imuABC* gene cassette are essential for ImuC to mediate TLS, and the deletion of any of these genes results in the depletion of DNA-damage induced mutagenesis (Galhardo *et al.*, 2005; Warner *et al.*, 2010). Accordingly, we also elucidated the importance of ImuA and ImuB in the alkylation damage tolerance. Our results demonstrated that the survival of both *P. aeruginosa* (Fig. 6A; Ref. III) and *P. putida* (Fig. 6B; Ref. III) Δ*alkA* cells deficient in either *imuA, imuB* or *imuC* was similar to that of the Δ*imuABCalkA* strains, suggesting their involvement in the same pathway. Moreover, almost complete restoration of MMS-tolerance observed in *P. putida* Δ*imuBalkA* strain by a chromosomal complementation with *imuB* gene suggested that all products of the cassette are needed for ImuC to function as a TLS polymerase to mediate protection against alkylation damage (Fig. S1; Ref. III).

If we go back to the temperature effect, then disappearance of ImuC functions, observed in *P. putida* at temperature above the growth optimum (at 37 °C), could be attributed to the ImuABC complex stability or to its possible interaction with DNA or other partners. It was demonstrated that temperature, aside from direct influence on the DNA structure, might also affect DNA-protein interactions (Driessen *et al.*, 2014). Still, the effect of the incubation temperature observed in

our study was unexpected. Both in *P. putida* and *P. aeruginosa* the importance of ImuC increases at lower temperature. In the case of *P. aeruginosa,* the increased DNA-damage response observed at lower temperature points at the decreased efficiency of repair systems, and therefore, ImuC might become more important for tolerance of alkylation damage. At the same time, such increased dependence on ImuC could potentially lead to the increased mutagenesis, associated with error-prone activities of this polymerase. As such, the ImuABC-mediated TLS might represent not only direct damage tolerance pathway, but also an adaptation mechanism to adverse growth conditions (Fig. 14).

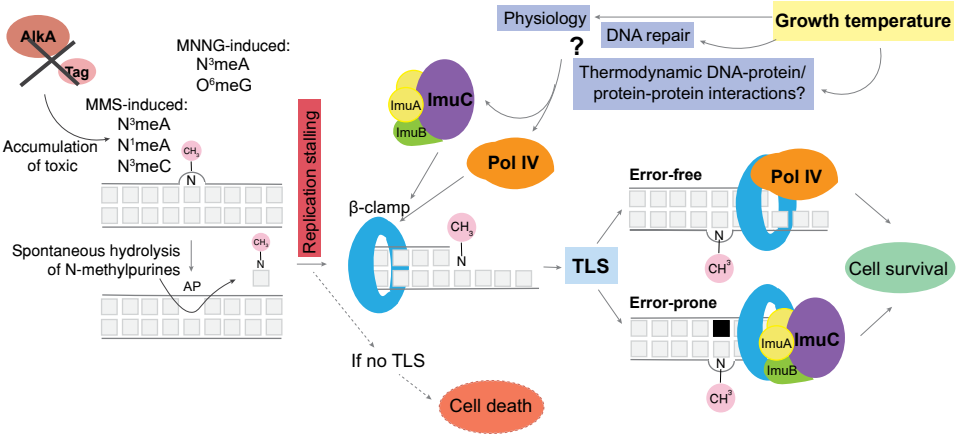

Figure 14. Alkylation damage tolerance in *Pseudomonas* species. Graphical representation of the main results. In both *P. putida* and *P. aeruginosa*, ImuABC and Pol IV are critical for the survival of bacteria in the presence of the MMS and MMNG-induced damage that accumulates in cells lacking DNA glycosylases AlkA and/or Tag. ImuABC appeared to be more important for the survival of AlkA-deficient bacteria in the case of MNNG-induced damage. If Pol IV-mediated TLS past alkylation damage is error-free, then mutagenesis induced by MMS in Pseudomonads is ImuC-dependent. The growth temperature of pseudomonads affects and modulates TLS function in response to alkylation damage. When *P. aeruginosa* cells are incubated at temperature below the growth optimum, ImuC becomes more important for the protection of cells against alkylation damage. When *P. putida* is incubated at 37 °C, the MMS-damage tolerance and MMS-induced mutagenesis become independent of ImuC function.

CONCLUSIONS

Our understanding of mechanism of mutation and genome stability has drastically changed over the last two decades when in all domains of life the translesion (TLS) DNA polymerases, mediating replication past DNA damage, were discovered. This unique class of polymerases not only ensures organism survival in the presence of DNA damage, but due to the intrinsic mutagenic nature also supplies cells with mutations, which can be a fuel for evolution, but also give rise to disease. In bacteria, TLS polymerases confer protection against various DNA damage, and error-prone DNA synthesis, associated with their low fidelity, contributes to fitness and adaptation and represents an important mechanism of antibiotic resistance and virulence. Therefore, understanding of the molecular mechanisms of TLS in more detail will provide us the strategy to combat pathogenic bacteria in the future.

In my work, I have characterized the *rpoB*/Rif^r test system for studying mutagenic processes in Pseudomonads. Next, I have investigated the role of TLS polymerases in spontaneous mutagenesis, contribution to chromosomal replication and UV-mutagenesis in bacteria lacking Polymerase I functions in *Pseudomonas putida.* In addition, I have investigated the involvement of TLS polymerases in tolerance of alkylation damage also in another representative of Pseudomonads, *Pseudomonas aeruginosa*. The major findings of my doctoral studies can be described as follows:

1. The results of my work uncovered that the time of Rif^r mutant isolation and growth temperature of bacteria can significantly affect the specificity of the spectra of mutations in the *rpoB*/Rif^r test system. Therefore, differential growth characteristics of Rif^r mutants, effect of the temperature on mutagenic processes and on the growth of mutants should be considered, when system is employed for comparative studies of mutagenesis in *Pseudomonas* species, which are commonly incubated at different temperatures. The results of my study demonstrate the existence of highly mutagenic hot spots in the *rpoB* sequence, and therefore, the translation of mutational specificity on the whole genome must be done with caution. Yet, the *rpoB*/Rif^r test system represents a robust, simple and reliable method that can be used to study spontaneous and induced mutagenesis in bacteria.
2. The analysis of the spectra of spontaneous mutations in the *rpoB* gene revealed that Pol IV might be responsible for the majority of A:T→G:C transitions in the wild-type bacteria that occur within the 5′-G<u>A</u>-3′ sequence context. On the other hand, Pol IV could also suppress mutations within the 5′-TC-3′context. Therefore, in *P. putida,* Pol IV might leave fingerprints in the genome and affect specificity of mutations at the specific sites. In Pol I-deficient bacteria, the increased spontaneous mutation rate might be associated with unrepaired mispairs introduced by the replicative Pol III during genome replication. In addition, TLS polymerases Pol IV and Pol II

could get access to DNA in the absence of Pol I, leading to the appearance and suppression of spontaneous mutations within specific sites of the genome.

3. ImuC, which is responsible for UV-induced mutagenesis in *M. tuberculosis* and *P. aeruginosa*, has a different role in *P. putida* cells. The results indicate that in response to UV-damage, ImuC could protect genome integrity of Pol I-deficient bacteria by mediating accurate bypass past some of UV-induced lesions, potentially suppressing A:T→G:C transitions. At the same time, ImuC could be responsible for the occurrence of A:T→T:A transversions. However, in the wild-type bacteria its activity might be either under strict regulation or the ImuC-mediated mutations could be efficiently repaired.
4. TLS polymerases in *P. putida* and *P. aeruginosa* have different roles in the protection of wild-type cells against methyl methanesulfonate (MMS)-induced damage. In *P. aeruginosa,* both Pol IV and ImuC appeared to be essential for the survival of bacteria in the presence of MMS. In *P. putida,* Pol IV and ImuC are important for the protection against MMS-induced damage only in actively growing cells. On the other hand, in both Pseudomonads TLS appeared to be important for the protection of bacteria against the N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-mediated damage, which in cells induces the formation of *N*- and *O*-methylation.
5. TLS is important for the protection of *P. putida* and *P. aeruginosa* cells against the alkylation damage that accumulates in bacteria lacking DNA-glycosylase mediated repair. ImuC appeared to be more important for the survival of AlkA-deficient bacteria in the presence of MNNG, suggesting its potential role in tolerance of MNNG-induced *O*-alkylation damage. In addition, the mutability of MMS in Pseudomonads is ImuC-dependent. On the contrary, Pol IV is involved in accurate MMS-induced damage bypass and in the suppression of mutagenic activity of ImuC.
6. The results of my studies revealed that the growth temperature of bacteria affects and modulates tolerance of alkylation damage. In the case of *P. aeruginosa,* incubation of bacteria at lower temperature decreases the efficiency (or changes regulation) of repair systems, leading to the accumulation of unrepaired lesions, which in turn increases the requirement of TLS in the protection of cells against alkylation damage. Incubation of *P. putida* cells at the temperature above its growth optimum leads to the disappearance of the ImuC-dependent MMS-mutability and MMS-induced damage tolerance.

SUMMARY IN ESTONIAN

Spetsialiseeritud DNA polümeraaside osalus mutageneesil ja DNA kahjustuste tolereerimisel pseudomonaadides

Erinevad endogeensed ja eksogeensed tegurid kahjustavad pidevalt meie genoomi terviklikkust. Kahjustused DNA ahelas aga blokeerivad DNA replikatsiooniprotsessi, mis võib negatiivselt mõjutada geneetilise informatsiooni edasikandmist tütarrakkudesse või osutuda rakkudele letaalseks. DNA kahjustuste eliminеerimiseks on rakkudes välja kujunenud mitmed DNA reparatsioonimehhanismid. Vaatamata sellele jäävad mõned kahjustused DNA ahelas parandamata. Selleks, et tagada geneetilise informatsiooni edasikandumine ja säilitamine, on igas organismis välja kujunenud DNA kahjustuste tolereerimismehhansimid, mis tagavad replikatsiooni lõpuleviimist, võimaldades rakkudel ellu jääda DNA kahjustuste olemasolul. Üheks selliseks DNA kahjustuse tolereerimise mehhanismiks on DNA kahjustusest ülesüntees (*translesion DNA synthesis*, TLS), mida viivad läbi spetsialiseeritud DNA polümeraasid. Need DNA polümeraasid on võimelised jätkama DNA sünteesi kahjustatud nukleotiidi kohalt, kus replikatiivne DNA polümeraas seiskub. Sõltuvalt DNA kahjustusest võib nende poolt läbiviidav kahjustusest ülesüntees (TLS) olla väga efektiivne ja täpne. Samas ebaspetsiifilise (mittesobiva) substraadi või kahjustamata DNA puhul on süntees tavaliselt vigaderohke. Seega, ühelt poolt võimaldab TLS rakkudel ellu jääda DNA kahjustuste korral, samas võib sellega kaasneda aga mutatsioonide hulga suurenemine rakus. Stressitingimustes suurenenud geneetiline mitmekesisus võib osutada kasulikuks, võimaldades kiiremat kohastumist ebasoodsate tingimustega. Samas võib liiga suur mutatsioonisagedus olla rakkudele letaalne (või olla seotud haiguse ja vähitekkega kõrgematel organismidel). Seega, selleks et tagada genoomi stabiilsus, peab TLS polümeraaside töö rakkudes olema täpselt reguleeritud, et hoida nende mutageenset potentsiaali kontrolli all. TLS ja spetsialiseeritud DNA polümeraasid mängivad bakteritel olulist rolli DNA kahjustuste tolereerimisel, kuid lisaks on näidatud nende osalust antibiootikumidele resistetsuse, virulentsuse ja nakkuspotensiaali (infektsioonivõime) kujunemisel. Seega võib TLS osutuda potentsiaalseks antibakteriaalse ravi märklauaks.

Meie teadmised TLS-ist bakterites baseeruvad peamiselt uuringutel, mis on teostatud klassikalise mudelorganismiga *Escherichia coli*. Sellel organismil on kirjeldatud kolm spetsialiseeritud DNA polümeraasi (Pol II, Pol IV ja Pol V), mis on võimelised viima läbi TLS-i ja on olulised erinevat tüüpi DNA kahjustuste tolereerimisel. Samas on *E. coli* osutunud viimaste aastate uuringute valguses võrreldes teiste organismidega erandlikuks, seda eriti DNA reparatsiooni ja DNA kahjustuste tolereerimismehhanismide osas. Näiteks puuduvad paljude bakterite genoomides geenid, mis kodeerivat mutageenset DNA polümeraasi Pol V. Selle asemel on neis organismides, mille hulka kuuluvad ka olulised patogeenid *Pseudomonas aeruginosa* (*P. aeruginosa*) ja *Mycobacterium tuberculosis* (*M. tuberculosis*), genoomis olemas *imuA-imuB-imuC* (endine *dnaE2*)

geenikasett, nn. "mutageensuse operon", mis kodeerib Pol V-le alternatiivset TLS süsteemi ImuABC. Selle mutageensuse operoni bioloogiline roll ei ole veel hästi arusaadav. Näiteks bakteris *M. tuberculosis* on ImuC vigutekitav DNA polümeraas ning kõik operoni kuuluvad geenid on vajalikud UV kiirguse- ja mitomütsiin C- poolt põhjustatud kahjustuste tolereerimiseks ja vastutavad indutseeritud mutageneesi eest. Lisaks sellele, tänu oma mutageensele potentsiaalile soodustab ImuC bakterite infektsioonivõimet ja antibiootikumide resistentsuse väljakujunemist. Samas ei ole aga *Streptomyces* tüvedel ImuC (DnaE2) vajalik UV-kiirguse poolt tekitatud kahjustuse tolereerumisel ja UV-mutageneesl. Pseudomonaadidel, mille rühma kuulub nii patogeenseid kui ka mittepatogeensid liike, mis on võimelised hõivama väga erinevaid elupaiku ja kiiresti kohanema muutuvate keskkonnatingimustega, on mutageenuse operoni funktsioone varem uuritud bakterites *P. aeruginosa* ja *P. putida*. Vaatamata sellele, et need bakterid kuuluvad ühte perekonda, leiti, et ImuC on bakteris *P. aeruginosa* vigutekitav polümeraas, mis vastutab UV-mutageneesi eest, samas kui mullabakteris *P. putida* oli näidatud, et ImuC toimib antimutaatorina, vähendades mutatsioonitekkesegadust nii UV-kahjustuste puhul kui ka statsionaarse faasi rakkudes. Lähtudes sellest sai minu töö eesmärgiks selgitada TLS polümeraaside funktsioone, eeskätt nende potentsiaalset rolli mutatsiooniprotsessides mudelorganismides *P. aeruginosa* ja *P. putida*.

1. Selleks, et uurida mutatsiooniprotsesse Pseudomonaadides, iseloomustasime kõigepealt kromosomaalset *rpoB*/Rif^r testsüsteemi, millega on võimalik tuvastada nii spontaanseid kui ka indutseeritud mutatsioone ja määrata mutatsioonide tekkesagedust eksponentsiaalse kasvufaasi rakkudes. See süsteem baseerub rifampitsiini resistentsuse tekkel mutatsioonide tagajärjel *rpoB* geeni. Selgus, et nii bakterite kasvutemperatuur kui ka mutantide isoleerimise aeg mõjutavad oluliselt mutatsioonispektrit. Ilmnes, et osad rifampitsiini resistentsust põhjustavad mutatsioonid *rpoB* geenis võivad mõjutada bakterite kasvukiirust ja omada pleitoroopset efekti erinevatel inkubeerimistemperatuuridel. Seega saab mutatsioonisagedusi ja spektreid võrrelda ainult samal temperatuuril kasvatatud bakteritüvedel. Samuti tuleb olla ettevaatlik *rpoB* geeni mutatsioonispektri tulemuste tõlgendamisel kogu genoomi kohta, kuna *rpoB* geenis on mõned mutatsiooniliselt kuumad punktid (*hot spot*).

2. Lisaks eelpoolmainitud ImuABC TLS süsteemile on pseudomonaadidel olemas veel kaks spetsialiseeritud DNA polümeraasi: B-perekonda kuuluv DNA polümeraas II (Pol II) ja Y-perekonda kuuluv DNA polümeraas IV (Pol IV). Uurides spetsialiseeritud DNA polümeraaside osalust spontaansete mutatsioonide tekkel bakteris *P. putida* leidsime, et kõige tugevama mutatsioonide sõrmejälje jätab mutatsioonispektris Pol IV. *rpoB* geeni mutatsioonispektri analüüsi põhjal ilmnes, et Pol IV soodustab peamiselt A:T→G:C mutatsioonide teket juhul, kui muteeruv A nukleotiid asub 5′-GA-3′ kontekstis. Samas 5′-TC-3′ DNA järjestuse kontekstis võib Pol IV võib ka mutatsioonide teket pärssida. Seega võib Pol IV *P. putida* rakkudes osaleda

DNA replikatsioonil, mõjutades mutatsioonide teket spetsiifilistes genoomi kohtades.

3. Replikatiivsed DNA polümeraasid III (Pol III) ja I (Pol I) viivad läbi genoomi replikatsiooni. Kui Pol III vastutab nii juhtiva kui ka mahajääva ahela sünteesi eest, siis Pol I osaleb mahajääva DNA ahela Okazaki fragmentide protsessimisel ning DNA reparatsiooniprotsessides. Selgus, et *P. putida* rakkudes suureneb Pol I puudumisel spontaansete mutatsioonide tekkesagedus. Samas ei olnud ükski spetsialiseeritud DNA polümeraasidest seotud mutatsioonisageduse suurenemisega Pol I puudumisel, kuigi nende Pol II ja Pol IV puudumisel muutus mutatsioonispekter. Seega võivad Pol II ja Pol IV Pol I puudumisel saada ligipääsu DNA replikatsioonile, mõjutades spontaansete mutatsioonide teket spetsiifilistes genoomi järjestustes.

4. Spetsialiseeritud DNA polümeraas ImuC, mis enamikus seniuuritud bakteriliikides on seotud UV-mutageneesiga, UV-kiiritatud Pol I-defektsetes *P. putida* rakkudes sarnast rolli ei omanud. Küll võib aga ImuC Pol I puudumisel potentsiaalselt pärssida A:T→G:C mutatsioonide teket ning soodustada A:T→T:A transversioonide teket. Samas Pol I olemasolul on ImuC DNA replikatsiooniga vähem seotud.

5. Alküleerivaid ühendeid leidub igal pool. Neid tekib nii rakulise metabolismi käigus (nt. metüülrühmadoonori S-adenosüülmetioniini toimel), kui esineb ka keskkonnas (vees, õhus, toidus). Need ühendid kahjustavad DNA-d (muuhulgas ka RNA-d jt makromolekule), modifitseerides lämmastikaluseid nukleiinhapetes ja põhjustades DNA replikatsiooni seiskumist. Selgus, et TLS mängib erinevatel Pseudomonaadidel erinevat rolli eksogeensete alküülkahjustuste tolereerimisel. Bakteris *P. aeruginosa* kaitsevad Pol IV ja ImuC mõlemad rakke metüül- metaansulfonaadi (MMS) poolt tekitatud kahjustuste eest. Samas mängib bakteris *P. putida* TLS olulist rolli MMS-i poolt põhjustatud alküülkahjustuste tolereerimisel ainult kasvavates (eksponentsiaalse kasvufaasi) rakkudes. Siiski kaitseb TLS nii *P. aeruginosa* kui ka *P. putida* rakke alküülkahjustuste eest, mis on tekitatud N-metüül-N′-nitro-N-nitrosoguanidiini (MNNG) poolt, mis lisaks lämmastiku aatomitele DNAs (N-alküleerimine) modifitseerib ka hapnikuaatomeid.

6. Nii ImuC kui ka Pol IV mängivad kriitilist rolli DNA alküülkahjustuste tolereerumisel, mis akumuleeruvad rakkudes, mis on defektsed DNA glükosülaaside suhtes, mis algatavad lämmastikaluste väljalõikereparatsiooni. Minu uurimistöö tulemused viitavad sellele, et ImuC võiks olla nii *P. putida* kui ka *P. aeruginosa* rakkudes oluline MNNG-poolt indutseeritud kahjustuste tolereerimisel. Lisaks viib ImuC läbi vigaderohket TLS-i alküleeritud DNA kahjustustelt, suurendades mutatsioonide tekkesagedust. Pol IV poolt läbiviidav TLS DNA alküülkahjustuste korral on aga väga täpne. Lisaks sellele ilmnes, et Pol IV avaldab antimutageenset efekti ImuC-le.

7. Bakterite inkubeerimistemperatuur mõjutab DNA alküülkahjustuste tolereerimist ja/või reparatsiooni efektiivsust. Kui *P. aeruginosa* rakud on kasvatatud temperatuuril, mis on optimaalsest madalam, siis TLS-i roll ja eriti ImuC olulisus suureneb alküülkahjustuste tolereerimisel. Kultiveerides bakteri *P. putida* rakke aga temperatuuril, mis on optimaalsest kõrgem, kaob nii ImuC-st sõltuv mutaatorfenotüüp, kui ka alküülkahjustuste eest kaitsev fenotüüp, viidates sellele, et kõrgemal temperatuuril ei osale ImuC DNA alküülkahjustuste tolereerimisel.

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Maybe the biggest thing I realized is that you have to live in the moment, dream and think big today, not tomorrow…because we only live once☺

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PUBLICATIONS

CURRICULUM VITAE

Name: Tatjana Jatsenko
Date of birth: 27.06.1986
Citizenship: Estonian
Current position: Researcher at Tervisetehnoloogiate Arenduskeskus AS, Tartu, Estonia

Education:

Since 2010	University of Tartu, Institute of Molecular and Cell Biology, PhD studies, molecular and cell biology
2008–2010	University of Tartu, Institute of Molecular and Cell Biology, MSc in gene technology (*cum laude*)
2005–2008	University of Tartu, Institute of Molecular and Cell Biology, BSc in gene technology
1993–2005	Tallinn Karjamaa Gymnasium

Language skills: Rusian, Estonian, English

Scientific activities

1. Main fields of research

1. Role of specialized DNA polymerases in genome stability and damage tolerance in Pseudomonds
2. Mutagenic processes in Pseudomonads
3. Reproductive genetics. Development of sequnecing-based technologies for preimplantation genetic screening and non-invasive prenetal testing (NIPT)

2. List of publications

1. Hindrek Teder, Mariann Koel, Priit Paluoja, Tatjana Jatsenko, Kadri Rekker, Triin Laisk-Podar, Viktorija Kukuškina, Agne Velthut-Meikas, Olga Žilina, Maire Peters, Juha Kere, Andres Salumets, Priit Palta, Kaarel Krjutškov (2018). TAC-seq: targeted DNA and RNA sequencing for precise biomarker molecule counting. bioRxiv 295253; doi: https://doi.org/10.1101/295253
2. Tšuiko, O.; Zhigalina, D.I.; Jatsenko, T.; Skryabin, N.A; Kanbekova, O.R; Artyukhova, V.G.; Svetlakov, A.V.; Teearu, K.; Trošin, A.; Salumets, A., Kurg, A.; Lebedev, I.N. (2018). Karyotype of the blastocoel fluid demonstrates low concordance with both trophectoderm and inner cell mass. Fertility and Sterility, XXX−XXX [in print].
3. Tšuiko, Olga; Jatsenko, Tatjana; Parameswaran Grace, Lalit Kumar; Kurg, Ants; Vermeesch, Joris Robert; Lanner, Fredrik; Altmäe, Signe; Salumets, Andres (2018). A speculative outlook on embryonic aneuploidy: Can

molecular pathways be involved? Dev Biol. 2018 Jan 31. pii: S0012-1606(17)30341-X. doi: 10.1016/j.ydbio.2018.01.014. [Epub ahead of print]
4. Sidorenko, Julia; Jatsenko, Tatjana; Kivisaar, Maia (2017). Ongoing evolution of *Pseudomonas aeruginosa* PAO1 sublines complicates studies of DNA damage repair and tolerance. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 797–799, 26−37.10.1016/j.mrfmmm.2017.03.005.
5. Jatsenko, Tatjana; Sidorenko, Julia; Saumaa, Signe; Kivisaar, Maia (2017). DNA polymerases ImuC and DinB are involved in DNA alkylation damage tolerance in *Pseudomonas aeruginosa* and *Pseudomonas putida*. PLoS ONE, 12 (1), e0170719.10.1371/journal.pone.0170719.
6. Martínez-García, Esteban; Jatsenko, Tatjana; KivisaSar, Maia; de Lorenzo, Victor (2015). Freeing *Pseudomonas putida* KT2440 of its proviral load strengthens endurance to environmental stresses. Environmental Microbiology, 17 (1), 76−90.10.1111/1462-2920.12492.
7. Sidorenko J., Jatsenko T., Saumaa S., Teras R., Tark-Dame M., Hõrak R., Kivisaar M. (2011). Involvement of specialized DNA polymerases Pol II, Pol IV and DnaE2 in DNA replication in the absence of Pol I in *Pseudomonas putida*. Mutat. Res. 717(1–2):63–77.
8. Jatsenko, T.; Tover, A.; Tegova, R.; Kivisaar, M. (2010). Molecular characterization of Rif(r) mutations in *Pseudomonas aeruginosa* and *Pseudomonas putida*. Mutation Research-fundamental and Molecular Mechanisms of Mutagenesis, 683, 1–2, 106−114.

3. Research grants and scholarships

FEMS Young Scientist and Graduate School in medicine and Biotechnology Meeting Grants for participation in:

„4th Microbial Genome Maintenance Meeting“, April 26–29, 2013, Oslo, Norway

„13th International Conference on Pseudomonas“, September 4–7, 2011, Sydney, Australia

„Microbial stress: from molecules to systems“, May 10–13, 2012, Belgirate, Italy

„Congress of Baltic Microbiologists“ (2013, 2014)

4. Other organizational and professional activities

Memberships:

Estonian Society for Microbilogy

Since 2017: Implementation of preimplanataion embryo diagnostics (PGD) and genetic diagnosis for aneuploidy testing (PGD-A) in Estonia.

CURRICULUM VITAE

Nimi: Tatjana Jatsenko
Sünniaeg: 27.06.1986
Kodakontsus: Eesti
Praegune töökoht: Tervisetehnoloogiate Arenduskeskus AS, Tartu, Estonia, teadur

Haridus:

Alates 2010	Tartu Ülikool, Loodus- ja täppisteaduste valdkond, Molekulaar- ja rakubioloogia õppekava, doktorantuur
2008–2010	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, MSc Geenitehnoloogia õppekava (*cum laude*)
2005–2008	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, BSc Geenitehnoloogia õppekava
1993–2005	Tallinna Karjamaa Gümnaasium

Keelteoskus: Vene, Eesti, Inglise

Teaduslik ja arendustegevus

1. Peamised uurimisvaldkonnad

1. Spetsialiseeritud DNA polümeraaside osalus mutatsiooniprotsessides Pseudomonaadides
2. Reproduktiivne geneetika. Sekveneerimispõhiste meetodite väljatöötamine embrüo siirdamiseelseks skriininguks (PGS) ja mitte-invasiivseks prenataalseks testimiseks (NIPT).

2. Publikatsioonide loetelu

1. Hindrek Teder, Mariann Koel, Priit Paluoja, Tatjana Jatsenko, Kadri Rekker, Triin Laisk-Podar, Viktorija Kukuškina, Agne Velthut-Meikas, Olga Žilina, Maire Peters, Juha Kere, Andres Salumets, Priit Palta, Kaarel Krjutškov (2018). TAC-seq: targeted DNA and RNA sequencing for precise biomarker molecule counting. bioRxiv 295253; doi: https://doi.org/10.1101/295253
2. Tšuiko, O.; Zhigalina, D.I.; Jatsenko, T.; Skryabin, N.A; Kanbekova, O.R; Artyukhova, V.G.; Svetlakov, A.V.; Teearu, K.; Trošin, A.; Salumets, A., Kurg, A.; Lebedev, I.N. (2018). Karyotype of the blastocoel fluid demonstrates low concordance with both trophectoderm and inner cell mass. Fertility and Sterility, XXX−XXX [in print].
3. Tšuiko, Olga; Jatsenko, Tatjana; Parameswaran Grace, Lalit Kumar; Kurg, Ants; Vermeesch, Joris Robert; Lanner, Fredrik; Altmäe, Signe; Salumets, Andres (2018). A speculative outlook on embryonic aneuploidy: Can

molecular pathways be involved? Dev Biol. 2018 Jan 31. pii: S0012-1606(17)30341-X. doi: 10.1016/j.ydbio.2018.01.014. [Epub ahead of print]
4. Sidorenko, Julia; Jatsenko, Tatjana; Kivisaar, Maia (2017). Ongoing evolution of *Pseudomonas aeruginosa* PAO1 sublines complicates studies of DNA damage repair and tolerance. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 797–799, 26−37.10.1016/j.mrfmmm.2017.03.005.
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6. Martínez-García, Esteban; Jatsenko, Tatjana; KivisaSar, Maia; de Lorenzo, Victor (2015). Freeing *Pseudomonas putida* KT2440 of its proviral load strengthens endurance to environmental stresses. Environmental Microbiology, 17 (1), 76−90.10.1111/1462-2920.12492.
7. Sidorenko J., Jatsenko T., Saumaa S., Teras R., Tark-Dame M., Hõrak R., Kivisaar M. (2011). Involvement of specialized DNA polymerases Pol II, Pol IV and DnaE2 in DNA replication in the absence of Pol I in *Pseudomonas putida*. Mutat. Res. 717(1–2):63–77.
8. Jatsenko, T.; Tover, A.; Tegova, R.; Kivisaar, M. (2010). Molecular characterization of Rif(r) mutations in *Pseudomonas aeruginosa* and *Pseudomonas putida*. Mutation Research-fundamental and Molecular Mechanisms of Mutagenesis, 683, 1–2, 106−114.

3. Saadud uurimistoetused ja stipendiumid

FEMS noore teadlase ja Biomeditsiini ja biotehnoloogia alase doktorikooli stipendiumid osalemiseks järgmistel konverentsidel:

„Protein Modification in Health and Disease“ Vihula Suvekool, Mai 25 – 28, 2011, Vihula, Eesti

„13th International Conference on Pseudomonas“, September 4 – 7, 2011, Sydney, Austraalia

„Microbial stress: from molecules to systems“, Mai 10–13, 2012, Belgirate, Itaalia

„4th Microbial Genome Maintenance Meeting“, Aprill 26–29, 2013, Oslo, Norra

„Congress of Baltic Microbiologists“ (2013, 2014)

4. Muu teaduslik organisatsiooniline ja erialane tegevus

Olen liige:

- Eesti Mikrobioloogide Ühendus

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