UiT
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Faculty of Science and Technology
Department of Chemistry

DNA metabolism in extremophiles

Structure-function studies of proteins involved in DNA repair and replication from Aliivibrio salmonicida and Deinococcus radiodurans

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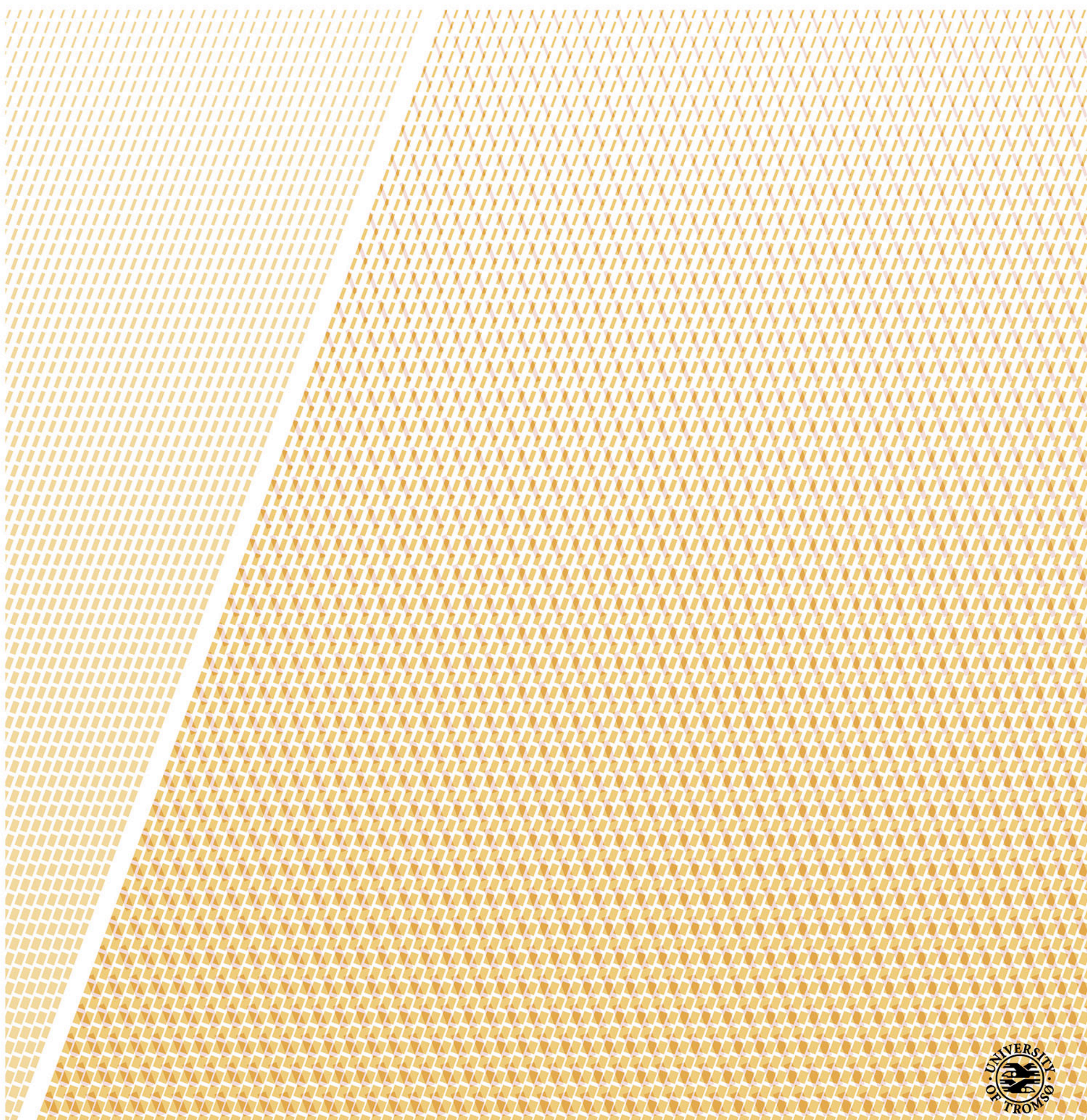
Kjersti Lian

A dissertation for the degree of Philosophiae Doctor – June 2015



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To Jonas, Anna and Sverre

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Kjersti Lian

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// Abstract

Background

DNA is a storage unit of genetic data, so in order to maintain this information intact changes in the DNA must be kept to a minimum. In this study, three enzymes involved in nucleotide pool sanitisation, DNA repair or replication from the two extremophiles *Aliivibrio salmonicida* and *Deinococcus radiodurans* were chosen as research models. The aim was to see how the enzymes contribute to their respective organisms´ extremophilic features and if they have gained individual functions to optimise the organisms DNA metabolism, in this respect.

Methods

The study was conducted by performing comparative biochemical and biophysical analysis of MutT from *A. salmonicida* (*As*MutT) and its mesophilic homologue *Vibrio cholerae* (*Vc*MutT). The crystal structure of the latter was determined and a comparative structural analysis was also performed. Further we determined the crystal structure of the DNA polymerase III β-subunit from *D. radiodurans* (*Dr*β-clamp) and analysed the biochemical properties N-terminally truncated Exonuclease III from *D. radiodurans* (*Dr*ExoIIIΔ22). For the latter a closer look into salt and $MgCl_2$ dependence during cleavage of damaged DNA was examined.

Results

Our findings show that *As*MutT possesses cold adapted properties with a higher catalytic efficiency than *Vc*MutT and lower activation energy due to lower activation enthalpy. However, *As*MutT exhibits an unexpected higher overall thermal stability than *Vc*MutT. By generating homology models of *As*MutT and comparing them to the crystal structure of *Vc*MutT, we were able to identify structural determinants, which we suggest explains our observed properties of AsMutT. The *Dr*β-clamp crystal structure was determined to 2.0 Å resolution, and found to be a homodimer forming the characteristic ring-shape of this protein. The calculated electrostatic surface potential revealed that the inner surface and the dimer interface had a more neutral charge distribution, indicating a less tight binding and thus a more efficient sliding on DNA compared to other structurally determined bacterial β-clamps.

The protein was co-crystallized with Cy5-labelled oligonucleotide DNA and the crystals appeared blue, however the DNA was not visible in electron density, thus confirming the weak protein-DNA interaction. *Dr*ExoIIIΔ22 possess AP-endonuclease, 3´-5´-exonuclease and 3´-diesterase activities. AP-endonuclease is dependent on the presence of magnesium and the exonuclease activity is salt sensitive in presence of $MgCl_2$. We believe the salt sensitivity might be of biological importance for *D. radiodurans* upon desiccation and a following increase in the intracellular salt concentration, forcing the enzyme specificity towards AP-endonuclease activity, which under these conditions will be more important for the maintenance of the genome stability.

Conclusion

Taken together, the three enzymes studied all show contributions of adaption to the organisms extremophilic properties, although further studies are needed to validate our hypothesises.

List of articles or publications

Manuscript I

MutT from the fish pathogen *Aliivibrio salmonicida* is a cold active nucleotide pool sanitization enzyme with an unexpected high thermostability. (2015) Lian K, Leiros HKS, Moe E. *FEBS Open Bio*. 5:107-16. doi:10.1016/j.fob.2015.01.006

Manuscript II

Crystal structure of the DNA polymerase III β subunit (β-clamp) from the extremophile *Deinococcus radiodurans*. (2015) Niiranen L, Lian K, Johnson KA, Moe E. *BMC Structural Biology,* 15:5, doi:10.1186/s12900-015-0032-6

Manuscript III

Biochemical characterisation of Exonuclease III from the extreme radiation and desiccation resistant bacterium *Deinococcus radiodurans.* (2015) Lian K, Timmins J, Moe E. *Manuscript*

Abbreviations

AMP	Adenosine monophosphate
AP-site	Apurinic/apyrimidinic or abasic site
APE1	Human AP-endonuclease, HAP1, Ref-1
APN1	AP-endonuclease from *S. cerevisiae*
A. salmonicida	*Aliivibrio salmonicida*
*As*Cat	*A. salmonicida* catalase
*As*EndA	*A. salmonicida* Endonuclease I
AST	Anionic salmon trypsin
ATP	Adenosine triphosphate
BER	Base excision repair
Cy5	Cyanine5
dGTP	diguanine triphosphate
DNA	Deoxyribonucleic acid
D. radiodurans	*Deinococcus radiodurans*
ds	Double strand
DSC	Differential scanning calorimetry
DSB	Double strand break
E_a	Activation energy
E. coli	*Escherichia coli*
EndoIII	Monofunctional glycosylase, Endonuclease III
EndoIV	AP-endonuclease, endonuclease IV
ExoIII	AP-endonuclease, Exonuclease III
GTP	Guanine triphosphate
Gy	Gray (derived unit of ionising radiation dose)
H	Planck constant, 6.63 × 10−34 J s
HR	Homologous recombination
IR	Ionising radiation
kb	Kilobase
k_B	Bolzmann constant, 1.38×10^{-23} J K^{-1}
k_{cat}	Enzyme reaction rate
k_{cat}/K_M	Katalytic efficiency

K_M	Concentration of substrate that leads to 50% V_{max}
LP	Long patch
MMR	Mismatch repair
MUG	Mismatch-specific uracil-DNA glycosylase
NAD^+	Nicotinamide adenine dinucleotide
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NIR	Nucleotide incision repair
NMN	Nicotinamide mononucleotidase
nt	Nucleotide
NTP	Nucleoside triphosphate
NTase	Nucleotidyltransferase
OB	Oligonucleotide/oligosaccharide binding
OH	Hydroxyl
PCNA	Proliferating cell nuclear antigen
PP_i	Inorganic pyrophosphate
R	Universal gas constant, 8.31 J K^{-1} mol^{-1}
ROS	Reactive oxygen species
S. cerevisiae	*Saccharomyces cerevisia*
SN	Single nucleotide
ss	Single strand
SSB	Single strand break
T	Temperature
T_m	Melting temperature
UNG	uracil-DNA N-glycosylase
UV	Ultraviolet
V. cholerae	*Vibrio cholerae*
5´-dRP	5´-deoxyribose phosphate
8-oxoG	7,8-dihydro-8-oxoguanine
β-clamp	DNA polymerase III β -clamp
$\Delta G^\#$	Gibbs free energy
$\Delta H^\#$	Enthalpy
$\Delta S^\#$	Entropy

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1. Introduction

1.1 DNA metabolism

Living cells are in constant motion and different cellular metabolic processes involving DNA are performed. DNA is a storage unit of genetic data, so in order to maintain this information intact and also for the sake of cell survival, changes in DNA must be kept to a minimum [1]. Luckily, cells can rely on a set of tightly regulated processes (DNA repair, replication, transcription and recombination) with a goal to maintain DNA uncorrupted. Here, we will focus on DNA repair and DNA replication.

1.1.1 DNA repair pathways

DNA damages are a common occurrence that compromises the functional integrity of DNA. The causative agents of these damages are several exogenous and endogenous sources as *e.g.* natural by-products of endogenic metabolic processes [2], cigarette smoke, chemotherapeutic drugs, UV light or ionising radiation (IR) [3]. It is estimated that more than 10,000 DNA bases are damaged daily in every human cell by oxidation, alkylation, deamination, depurination, and depyrimidation [4-6]. If damaged DNA is left unrepaired, it can generate cell-cycle arrest in all phases and inhibition of the cells metabolism as *e.g.* replication, which can cause cell death. A more persistent DNA damage can be caused by mutations, which ultimately are associated with cancer, early aging and inborn diseases. To counteract these potential damages cells have multiple complex cellular defence mechanisms that repair or prevent damage to occur. These networks of complementary DNA repair pathways include nucleotide excision repair (NER), mismatch repair (MMR), double strand break repair (homologous recombination (HR), and non-homologous end joining (NHEJ)) and base excision repair (BER) (**Figure 1**).

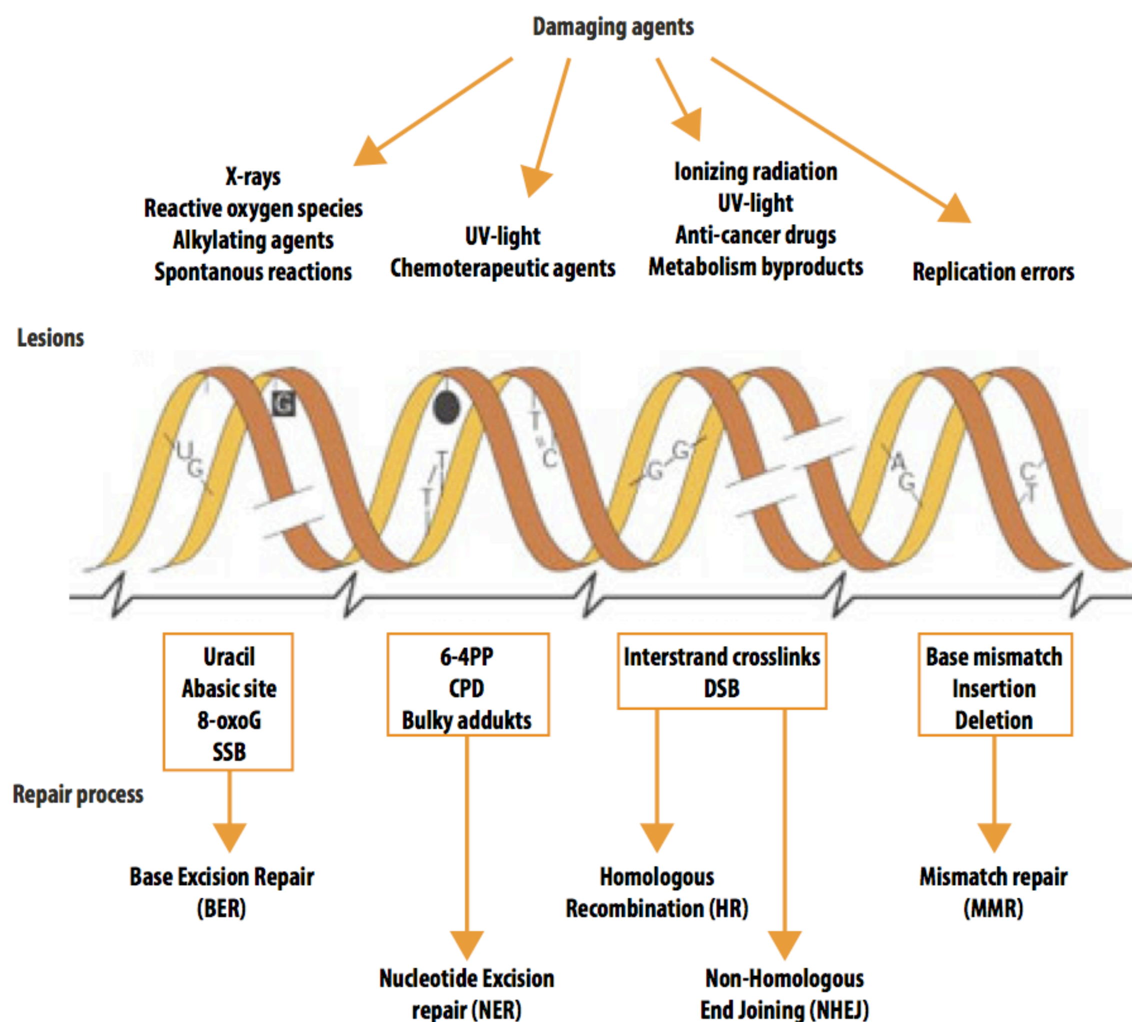

Figure 1 DNA-damaging agents, DNA lesions, repair mechanisms and consequences. Overview of different types of DNA damage that can occur in cells caused by endogenous and exogenous damaging agents. These damaging agents can cause single or double strand breaks in the DNA, base modifications (*e.g.* uracil, abasic site, 8-oxoG), helix-distorting bulky lesions in form of cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs), cross links of DNA strands, base mismatch, insertions and deletions. Several complex networks of complementary DNA repair pathways are available to repair each class of DNA damage. These include base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), non-homologous end joining (NHEJ), and mismatch repair (MMR). Figure modified from [7].

In the NER pathway the enzymes involved act upon a number of different DNA damaged sites caused by both UV-light and chemotherapeutic agents [3]. The two most common substrates of NER are the bulky DNA adducts induced by UV-light: (6-4) photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs) [8]. Defects in this pathway can cause the well-studied photosensitive syndromes Xeroderma pigmentosum (XP),

Cockayne´s syndrome (CS) and Trichothiodystrophy (TTD) (Reviewed in [3]). Enzymes in MMR remove mismatched bases from DNA caused by replication errors, as well as both spontaneous and chemical induced base deamination, oxidation, and methylation. Defective MMR proteins can cause the autosomal dominant syndrome known as Lynch syndrome or hereditary nonpolyposis colorectal cancer (HNPCC) [9]. Double strand breaks (DSBs) are a major threat to the genome and if left unrepaired can cause cell death or lead to cancer in multicellular organisms [10]. DSBs are induced by both exogenous agents as IR and various anti-cancer drugs, and by endogenous free radicals that are by-products of oxidative metabolism. As mentioned above, DSBs can be repaired by one of two mechanisms (HR or NHEJ). In dividing cells where a sister chromatid is available HR is favoured to ensure precise repair. In non-dividing cells, a homologous strand is not accessible, so instead the NHEJ pathway repairs the damage by joining the broken ends and thereby makes sure the chromosome is kept intact at the expense of precision [11, 12] (pathways are reviewed in [8]). BER is the main protective mechanism against base damages in DNA caused by natural by-products of endogenic metabolic and immune processes [2, 6], and exogenous sources, as *e.g.* cigarette smoke, chemotherapeutic drugs, UV light or ionising radiation [3, 6]. BER is described in detail below.

1.1.2 Base Excision Repair (BER)

In 1974, Tomas Lindahl identified the first uracil-DNA N-glycosylase (UNG), leading to the discovery of the BER pathway [13]. The repair process in BER takes place in four core steps as described in [14]: Excision, incision, end-processing and repair synthesis, which includes gap filling and ligation. The process is illustrated in **Figure 2**, exemplified by uracil repair.

The enzymes involved in BER are highly conserved during evolution [15]. In bacteria, UNG and other enzymes, which cleave the N-glycosylic bond between mismatched or modified base in DNA are called DNA glycosylases and initiate the first step of BER. DNA glycosylases are small monomeric proteins recognising damaged and mispaired bases in DNA without requirement for co-factors [16]. These base lesions do not cause major helix distortions [14], so it is believed that the glycosylases binds and diffuse along intact DNA and scan for lesions [17].

The BER pathway has two alternative routes; the single nucleotide (SN), also referred to as short patch, and the long patch (LP) repair pathways (**Figure 2**). The SN-repair pathway is suggested to be the main road in BER and has several proteins that are equally efficient in proliferating and non-proliferating cells [14]. Here, only a single nucleotide is removed when polymerase I incorporates the correct single nucleotide while its lyase activity removes the 5´-dRP [18], generating the 5´-phosphate end required for ligation, and the nick in the DNA is thereafter sealed by a DNA ligase [14, 19, 20]. If the 5´-dRP moiety is oxidised or reduced the lyase activity of Polymerase I is inhibited and can not remove the modified 5´ sugar, and the LP-BER is activated [21, 22]. Polymerase I can fill the one base gap and synthesise 2-10 bases in DNA by displacing the DNA downstream of the initial damage site putting the modified 5´dRP into a 2-10 nt 5´-flap intermediate that can be cleaved by a 5´-flap endonuclease and further ligated by a ligase [19]. All of the enzymes involved represent essential steps in BER and are conserved from *E. coli* to humans [6].

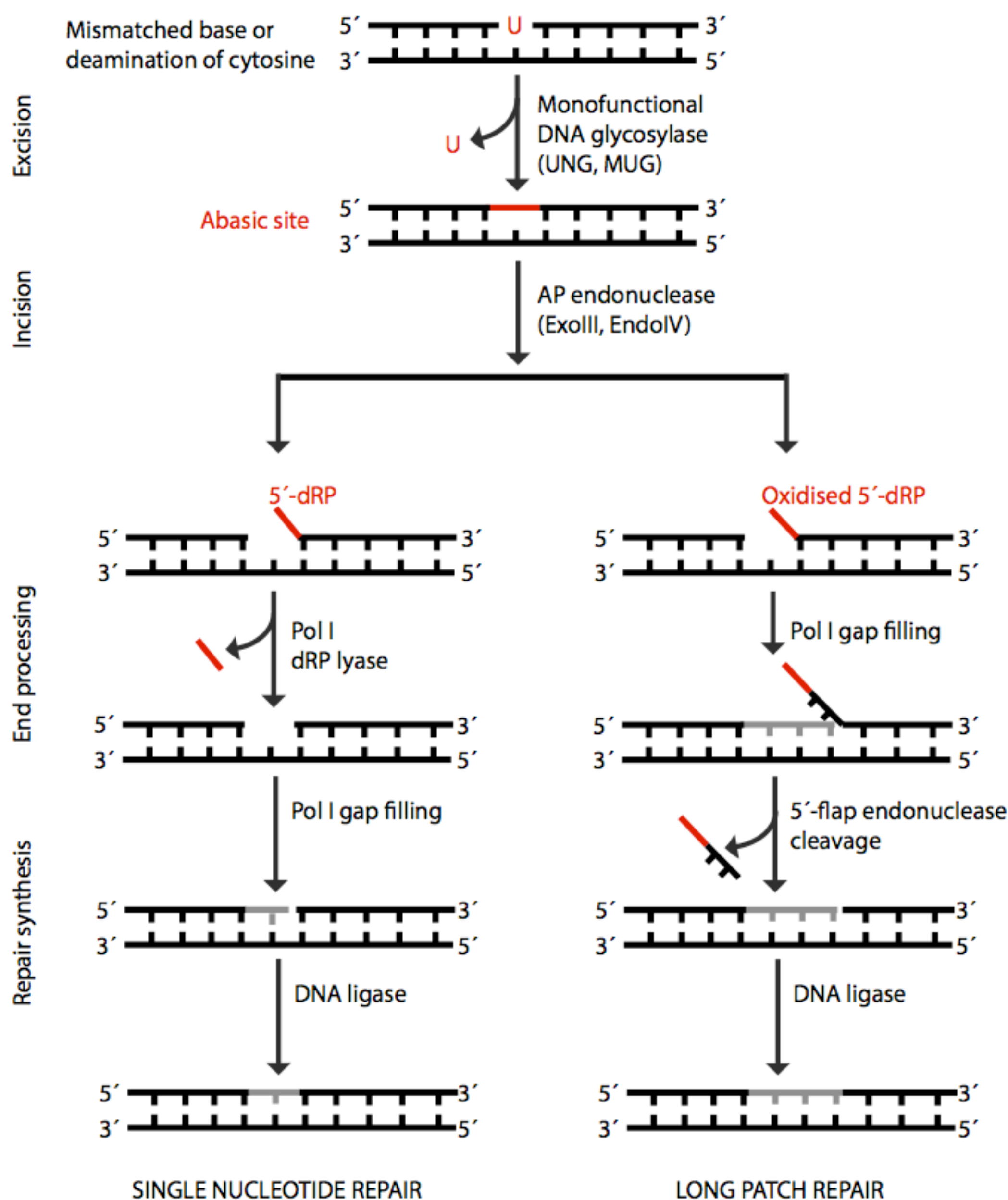

Figure 2 BER pathway is initiated by the removal of a damaged base by a glycosylase, and each subsequent repair step produces another form of DNA lesion until repair is completed. Here, a cytosine has been deaminated or a uracil has been mis-incorporated in DNA during translation. This lesion is recognised by a glycosylase (UNG or mismatch specific uracil-DNA glycosylase (MUG)) leaving an AP-site recognised by an AP-endonuclease (ExoIII, EndoIV). The AP-endonuclease cleaves 5´ to the abasic site leaving a 3-OH and a 5-deoxyribosephosphate (dRP) group. The polymerase I further removes dRP and inserts the correct nucleotide before the remaining nick is sealed by a ligase (short nucleotide repair). If the dRP-group is oxidised or reduced, the lyase activity of Polymerase I is inhibited and the LP-BER is activated. Polymerase I can fill the one base gap and synthesise additional bases in DNA by displacing the DNA downstream of the initial damage site putting the modified 5´dRP into a 2-10 nt 5´-flap intermediate that can be cleaved by a 5´-flap endonuclease and further ligated by a ligase.

DNA glycosylases can be divided into two classes; monofunctional and bifunctional (**Figure 3**). Monofunctional glycosylases cleave only the glycosidic bond between N and C1´ using water as a nucleophile, and yields an apurinic/apyrimidinic (AP-site) or abasic site. This site is further processed by an AP-endonuclease, cleaving the DNA on the 5' side of the AP-site leaving a 3´-OH and 5´-deoxyribose phosphate (5´-dRP) termini [23]. If the glycosylase is bifunctional it cleaves the DNA on the 3' side of the AP-site by β- or β-δ-elimination itself, after removal of the damaged or modified base, and yields a 5´-phosphate and 3´-lesions as 3´-unsaturated aldehyde or 3´-phosphate, respectively [24, 25] (Illustrated in **Figure 3**). These 3´-lesions block further repair by DNA polymerase and ligase [26], and are therefore removed by enzymes with phosphodiesterase and phosphatase activity like the AP-endonucleases Exonuclease III and Endonuclease IV to yield a 3´-hydroxyl group.

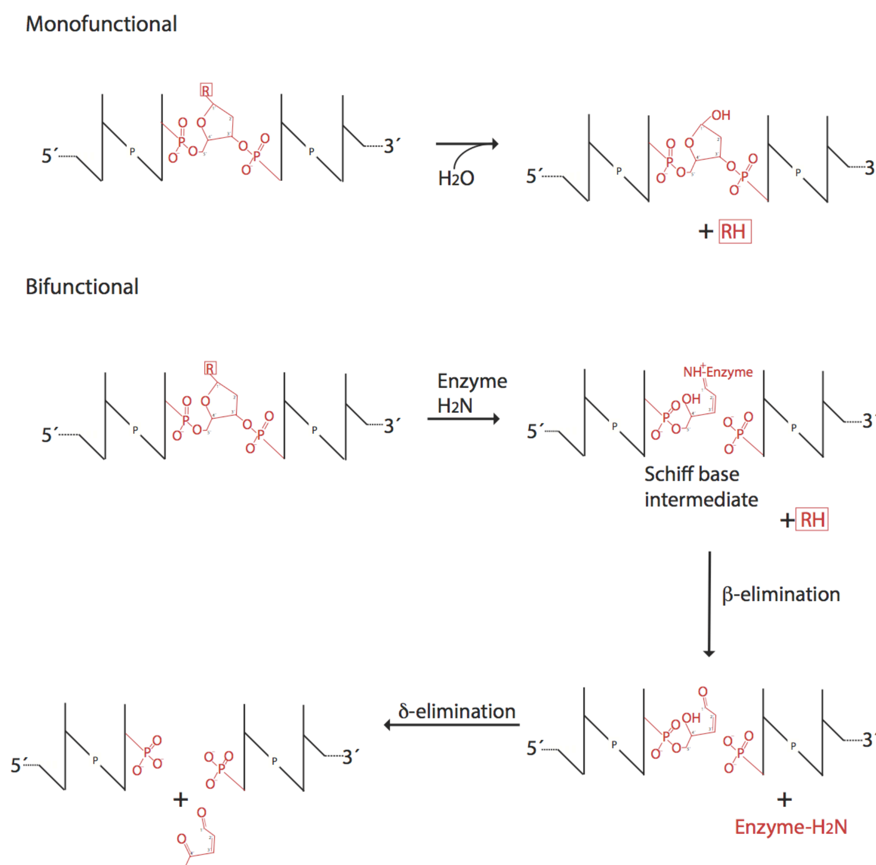

Figure 3 N-Glycosidic bond hydrolysis in DNA by monofunctional and bifunctional DNA glycosylases. Monofunctional glycosylases cleave the glycosidic bond between N and C1´ using water as a nucleophile, and yields an abasic site. The bifunctional glycosylases cleaves the DNA on the 3' side of the AP-site by β- or β-δ-elimination and yields 3´-lesions as 3´-unsaturated aldehyde or 3´-phosphate, respectively. R represents any residue. The figure is modified from [27].

Several different bacterial DNA glycosylases have been identified and cloned, all of which recognise and repair unique lesions typically resulting from oxidation damage, alkylation damage and deamination of bases (reviewed in [16, 17, 28]) (**Table 1**). Although the residues used to recognise lesions differ between the glycosylases, they all bind to the minor groove of the DNA where they make a kink at the site of damage and flip the damaged base out of the major groove and into the active site pocket of the enzymes [29].

Table 1 Overview of DNA glycosylases from *E. coli*

DNA glycosylase	*E. coli* gene	Causative agent for damage	Specificity	AP lyase
Uracil-DNA N-glycosylase	*UNG*	Mismatched base or deamination	U in ss/dsDNA	No
Mismatch specific uracil-DNA glycosylase	*MUG*	of cytosine		No
Formamidopyrimidine DNA glycosylase	*Fpg* (MutM)	Reactive oxygen species (ROS)	Oxidised purines (8-oxoG in 8-oxoG:C)	Yes (β/δ)
Endonuclease III	*Nth* (EndoIII)		Oxidised pyrimidines	Yes (β)
Endonuclease VIII	*Nei* (EndoVIII)		Oxidised pyrimidines and purines	Yes (β/δ)
3-methyladenine DNA glycosylase II	*AlkA*	Alkylation, deamination of adenine	3-MeA, 7-MeG, Hypoxanthine	No
3-methyladenine DNA glycosylase I	*Tag*	Alkylation	Ring-alkylated purines	No
Adenine-specific mismatch-DNA glycosylase	*MutY*	Mismatched base	A in A:8-oxoG and A:G	No
Nucleoside triphosphate pyrophosphohydrolase	*MutT**		Hydrolyse 8-oxo-GTP/dGTP to 8-oxo-GMP/dGMP	No

* Mutator protein

1.1.2.1 Oxidative damages and MutT

A common oxidative DNA damage is the highly mutagenic guanine derivative 7,8-dihydro-8-oxoguanine (8-oxoG) in DNA or 8-oxo-dGTP in the nucleotide pool, which later can be misincorporated into DNA [30, 31]. 8-oxoG base pairs with both adenine and cytosine in DNA, which consequently can induce A:T to C:G and G:C to T:A mutations (**Figure 4**). In *E. coli* three enzymes have been found that protect the organism against 8-oxoG-lesions [6]. MutY recognises the mismatched 8-oxoG:A base pair in DNA and

removes the adenine, making room for a cytosine to be paired with 8-oxoG. MutM (Fpg) has the ability to recognise paired 8-oxoG:C, and excise the oxidised guanine preventing mutation [32] (**Figure 4**). Although it is not a DNA glycosylase, the mutator protein MutT plays an important role in the cell's sanitisation system to prevent incorporation of 8-oxoG into DNA by hydrolysing the 8-oxoG containing deoxyribose or ribonucleoside triphosphates to their corresponding monophosphates and inorganic pyrophosphate [33] through a nucleophilic attack at the β-phosphorus atom (**Figure 4**) [30, 31].

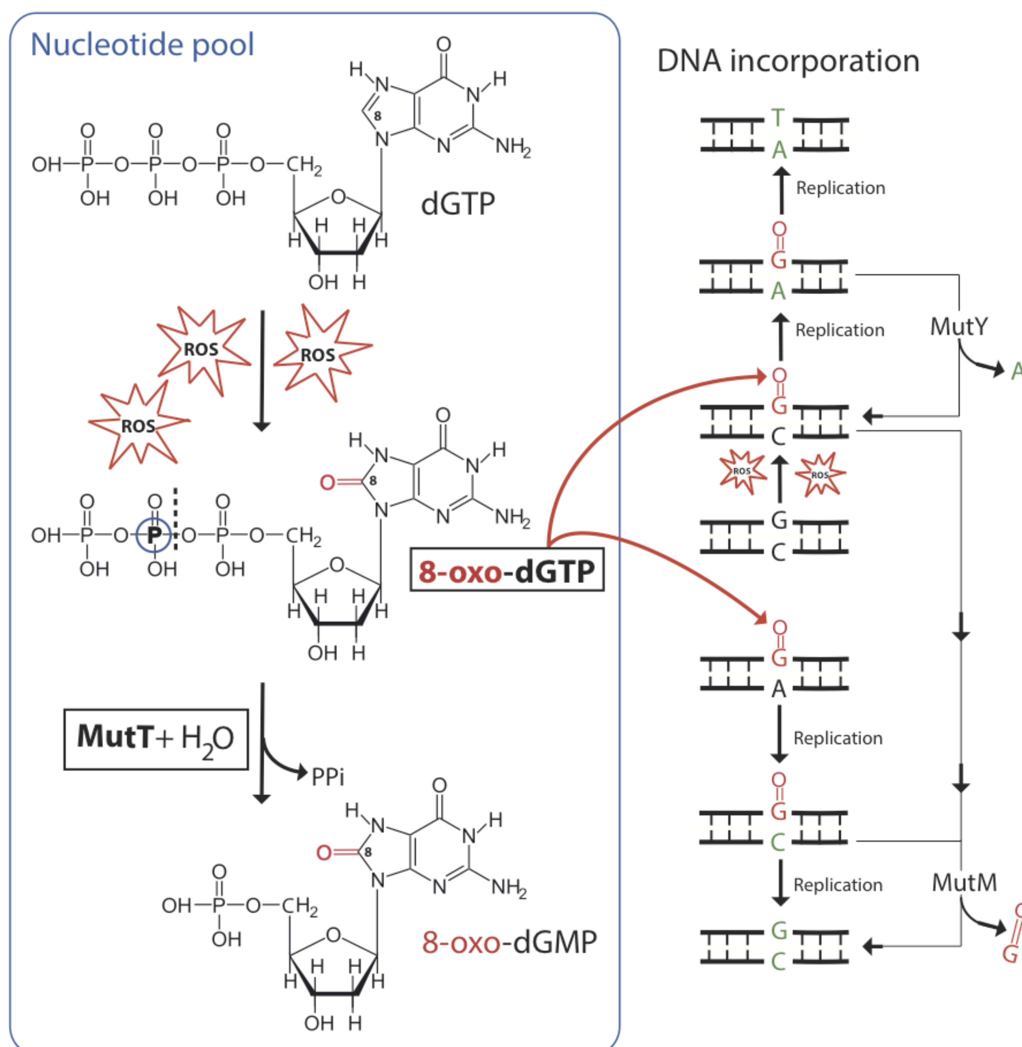

Figure 4 A common damage generated by ROS is the oxidation of guanine, and it is formed in DNA either by direct guanine oxidation, or by incorporation of 8-oxoG from the nucleotide pool. Herein, MutT catalyses the hydrolysis of 8-oxo-GTP by nucleophilic substitution at the β phosphorus site, yielding 8-oxo-dGMP and inorganic pyrophosphate. When 8-oxoG is incorporated in DNA opposite adenine or cytosine, the organism is equipped with several mechanisms to counteract the mutagenic effects of 8-oxoG. Here, the glycosylases from base excision repair pathway MutM and MutY function to prevent T:A to G:C and G:C to T:A mutations respectively. Figure from manuscript I [34].

MutT is a monomeric protein of about 15 kDa in size. It is widespread in nature and representatives are found in eukaryotes, prokaryotes, archeae and viruses [35]. It belongs to a superfamily of enzymes shown to require divalent cations for activity [36, 37]. Members of this family are recognised by a highly conserved 23-residue motif, or Nudix box, $GX_5EX_7REUXEEXGU$, where U is a bulky hydrophobic or aliphatic residue and X is any residue [38, 39]. The Nudix box forms a loop-α-helix-loop structural motif [40] that constitutes the active site.

*Ec*MutT was the first Nudix hydrolase to have its structure determined and mechanism studied in detail [41, 42]. The structure of human MutT (MTH1) was solved by NMR [43], and although *Ec*MutT and MTH1 share poor sequence similarity outside the conserved Nudix motif, they adopt a similar globular and compact structure with the parallel portion of a β-sheet sandwiched between two α-helices, forming an α + β fold. The structure study of *Ec*MutT [42] revealed that the loops between β2–β3 are flexible, but upon substrate binding the enzyme adopts a more closed conformation, in which these loops fold over the active site interacting with each other and become well defined in the *Ec*MutT complex structures (**Figure 5A**).

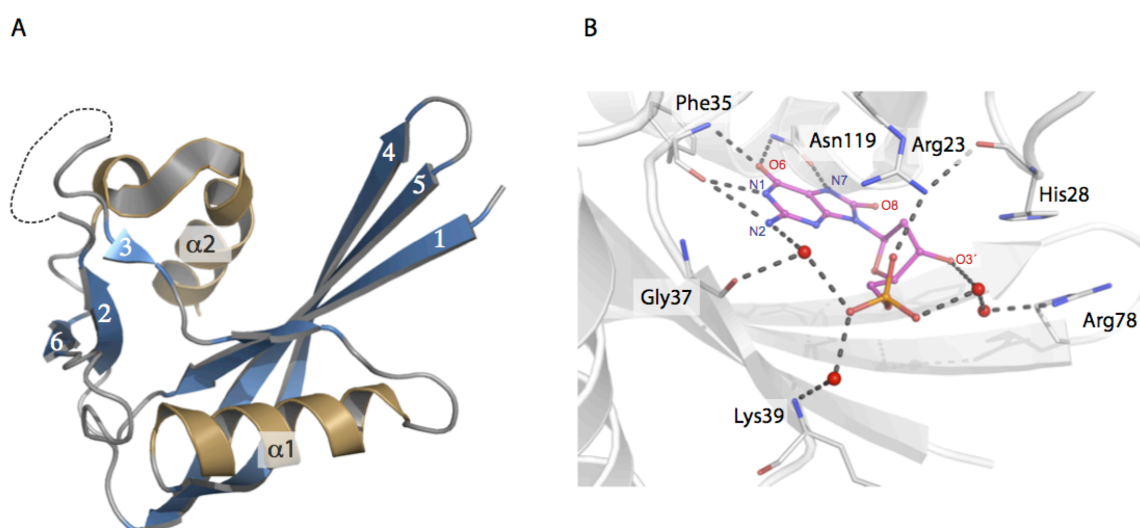

Figure 5 (A) Structure of *Ec*MutT-Apo (PDB: 3A6S) contains two α-helices and five β-sheets. The loop connecting β2 and β3 is not visible in the electron density map and is here represented by a dotted line. α-helices are in sand, β-sheets in sky-blue and loops in grey. (B) *Ec*MutT bound to the substrate 8-oxo-dGMP (PDB: 3A6T). Important residues involved in the interaction are shown. Figures are made in Pymol (The PyMOL Molecular Graphics System, Version 1.4 Schrödinger, LLC).

MutT enzymes recognise preferentially 8-oxo-dGTP over dGTP [44], and structural studies of *Ec*MutT have demonstrated that it undergoes conformational changes upon substrate binding [41, 42, 45]. The analyses have suggested that Asn119 is especially important for the affinity between *Ec*MutT and 8-oxo-dGMP. Saraswat *et al.* (2004) [46] described this by performing an N119A-mutation, which led to a 1650-fold decrease in the affinity of the enzyme for 8-oxo-dGMP.

Other interesting and seemingly important residues in substrate binding includes the guanidinium group of Arg23 that interacts with the phosphate group of 8-oxoG through a metal mediated interaction with Gly37 O, and through waters to Arg78 NZ and Lys39 N atoms. The main chain atoms of Phe35 make three different hydrogen bonds to N2, N1 and O6. From the O3' atom of the deoxyribose there is a direct bond to His28 (ND1) and a water-mediated interaction to Arg78 NZ (**Figure 5B**).

The interest in studying MutT enzymes have drastically increased based on recent findings that inhibitors of MTH1 can be used as anti-cancer drugs [47, 48]. By targeting MTH1 with inhibitors the cancer cells will not be able to clean up the oxidised nucleotide pool and thus increase the cancer cell's sensitisation to cancer treatment. This finding also opens for investigation of use of MutT inhibitors for treatment of infections by pathogenic bacteria as an alternative to antibiotics.

1.1.2.2 AP-endonucleases

AP-sites are the most frequent type of lesion found in DNA [49] and they are removed by two different classes of enzymes belonging to the BER pathway [24]. Class I corresponds to bifunctional DNA glycosylases, which have both glycosylase and AP lyase activity. They cleave the AP-site by β-elimination or β-δ-elimination and yield 3´-blocking lesions as 3´-aldehyd (**Figure 6**) or 3´-phosphate, respectively [24, 25]. Such lesions also arise directly as a result of oxidative damage [50, 51] and block further repair by DNA polymerase and ligase [26] and can promote chromosome instability [52]. Class II includes enzymes where the main activity is the hydrolytic AP-endonuclease activity where the DNA backbone is cleaved 5´ to an AP-site to produce 3´-OH groups and 5´-deoxyribosephosphate (5´-dRP) residues [24] (**Figure 6**). Class II AP-endonucleases are further divided into two families based on sequence similarity. In family 1 (also classified as *Nfo*-family), we find amongst others Endonuclease IV from *Escherichia coli*

(*E. coli*) [53] and APN1 from *Saccharomyces cerevisiae* [54]. Family 2 (*Xth*-family) includes ExoIII from *E. coli* (*Ec*ExoIII) [55] and APE1 enzyme from human [56]. In *E. coli*, the latter is the main AP-endonuclease and the EndoIV is only expressed when induced by superoxide anion generators [57]. APN1, on the other hand, is the essential AP-endonuclease in *S. cerevisiae* [58].

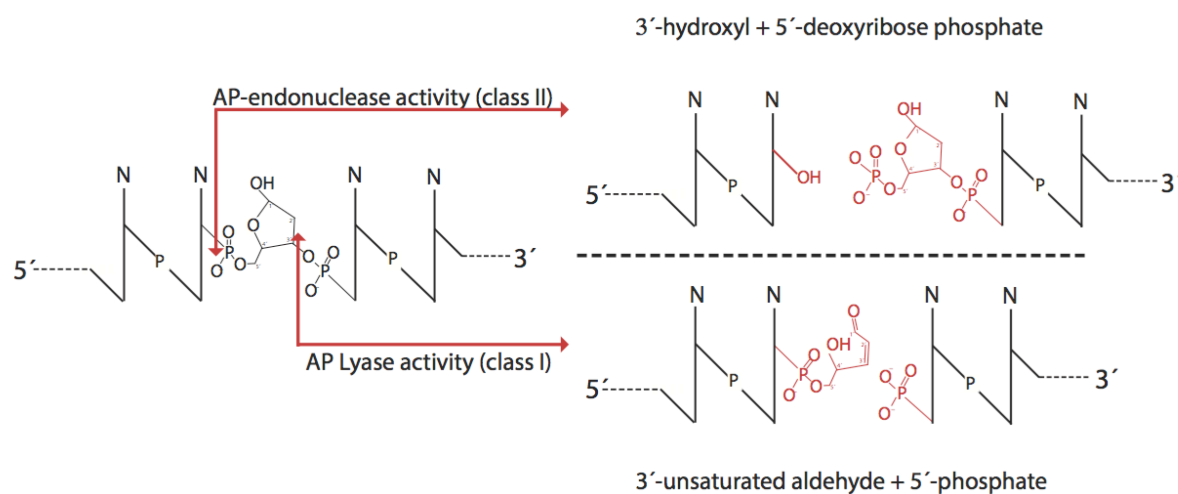

Figure 6 Structure of an abasic site and the cleavage position for class I AP lyase and class II AP-endonuclease. Here, Class I AP lyases cleave the AP-site by β-elimination to yield a 3´-unsaturated aldehyde. Class II AP-endonucleases have a hydrolytically AP-endonuclease activity where the DNA backbone is cleaved 5´ to an AP-site to produce 3´-OH groups and 5´-deoxyribosephosphate termini. N represents any base. The figure is modified from [52].

*Ec*ExoIII and APE1 can both cleave 3´-unsaturated aldehyde-groups generated by class I AP-endonucleases to yield 3´-hydroxyl (OH) groups (3′-phosphodiesterase activity) (**Figure 7A**) [24, 51] and also have the ability to degrade the RNA strand of an RNA-DNA hybrid duplex (RNAse H-activity) [59-61]. *Ec*ExoIII can act as a phosphatase by cleaving on the 5´ side of the 3´-phosphate groups [62] (**Figure 7B**) and in addition it has 3´ - 5´-exonuclease activity on double stranded DNA [63], where linear DNA is degraded from both 3´-ends until approximately 50% is digested [63]. These activities are present, though reduced, in APE1 [64]. APE1 exhibits nuclease incision repair (NIR) activity allowing it to incise DNA at oxidative base lesions without the presence of glycosylases. It is the unique N-terminal tail of APE1 that regulates the NIR-activity [65], seeing that the gene expressing *Ec*ExoIII (*XthA*) has a 63 nt shorter N-terminal tail than APE1, which

most likely explains why *Ec*ExoIII lacks NIR-activity. This pathway serves as a backup-repair pathway to BER, and the cleavage provides the correct ends for polymerase and ligase to continue synthesis.

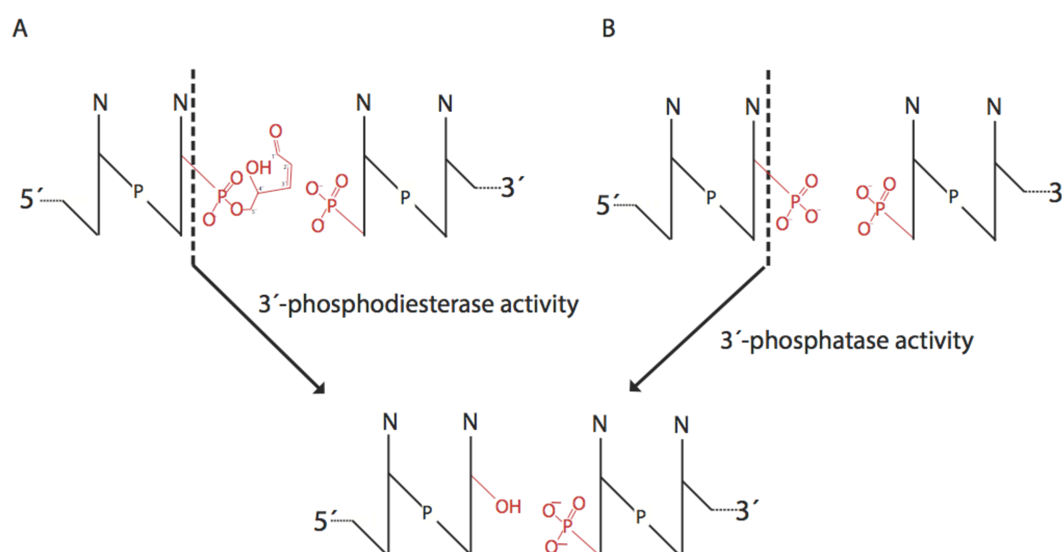

Figure 7 Structural overview over 3´-repair diesterase activity that yields 3´-OH groups. (A) 3′-phosphodiesterase activity cleaves 3´-unsaturated aldehyde termini and (B) 3´-phosphatase activity cleaves 3´-phosphate groups generated by class I AP lyases´ β-elimination and β-δ-elimination, respectively. N represents any base.

Crystal structures of *Xth*-family AP-endonucleases have been determined from various sources [24, 66-70], but the *Ec*ExoIII and APE1 are the most studied. *Ec*ExoIII and APE1 enzymes form a four-layered α/β-sandwich motif [71, 72] (**Figure 8**). They both show divalent cation dependence for AP-site recognition and cleavage at the active site [24]. The active site is located in a pocket on top of α/β-sandwich with surrounding loop regions. Most studies on AP-endonucleases involving DNA interaction have focused on abasic-site cleavage, but although AP-endonucleases recognise many different types of lesion it is believed that they employ a common active site, using the residues involved to varying extents [67, 73]. Solving the crystal structures of APE1 and *Ec*ExoIII have revealed several conserved residues important for metal binding and activity (APE1/*Ec*ExoIII: Asp283/229, His309/259, Asp210/149, Asn212/151, Asp308/258, Glu96/34) [59, 71, 73]. The main structural differences are found in surface loop regions where small residue insertions and deletions are apparent [71].

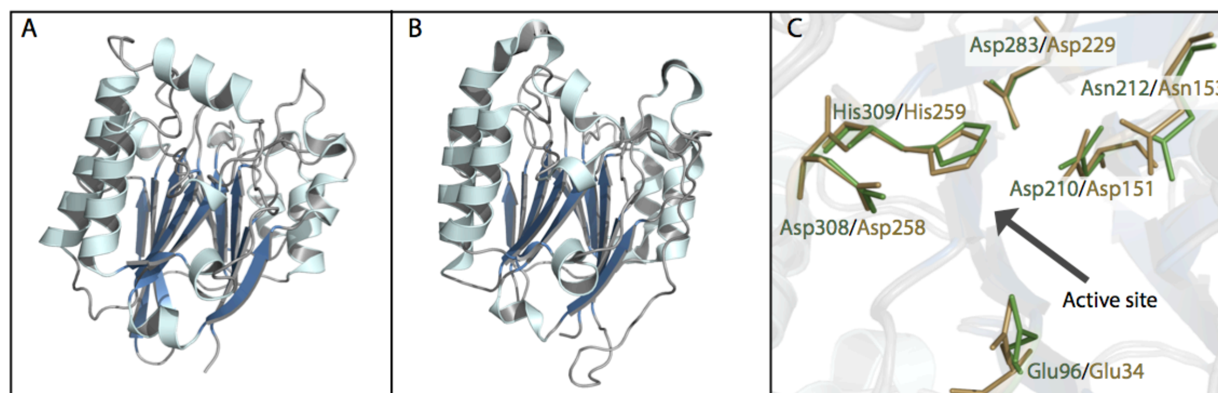

Figure 8 An overall view of the structure of (A) *Ec*ExoIII (PDB: 1AKO) and (B) human APE1 (PDB: 1DEW) to illustrate the four-layered α/β-sandwich motif. (C) The active site with residues found to coordinate Mg^{2+} binding and for the activation of the catalytic mechanism of phosphodiester bond cleavage. α-helices are in skyblue and β-sheets in pale cyan. APE1 residues are in green and *Ec*ExoIII ones in sand. Figures are made in Pymol (The PyMOL Molecular Graphics System, Version 1.4 Schrödinger, LLC).

1.1.2.3 DNA polymerase I

Gaps resulting from the activities of AP-endonucleases are filled principally by the multifunctional enzyme polymerase I in prokaryotes [15, 74], and it is also involved in post-replication repair of DNA gaps and double-strand breaks. Polymerase I is part of the DNA polymerase A family and is a single polypeptide made of two fragments with three separate enzymatic activities: 5´-3´-polymerase, 3´-5´-exonuclease and 5´-3´-exonuclease activity [75]. The largest fragment, known as the Klenow fragment, contains two sub-domains. The 5´-3´-polymerase activity that is located in the C-terminal part and catalyses the addition of dNTPs to the 3´end of the DNA chain or primer and the 3´-5´-exonuclease activity located in the N-terminal end proofreads for possible mistakes by the polymerase. The smaller fragment contains the 5´-3´-exonuclease activity, which removes unwanted nucleotides in advance of the growing DNA, and also removes the RNA primer from the lagging strand and fills in the necessary nucleotides of the Okazaki fragments in a 5' to 3' direction.

1.1.2.4 DNA ligase

DNA ligases play essential roles in DNA replication, recombination and repair. They join broken DNA strands by catalysing the formation of a phosphodiester bond between the

3' hydroxyl end of one strand and the 5' phosphate end of another. Ligation is performed in three steps (**Figure 9**): (1) a covalent enzyme-adenylate intermediate is formed when an active-site lysine attacks the adenosine monophosphate (AMP) moiety of adenosine triphosphate (ATP) or nicotinamide adenine dinucleotide (NAD^+) releasing pyrophosphate [33] or nicotinamide mononucleotide (NMN), respectively, and creating an activated state of the enzyme. (2) The AMP-group is then transferred to the 5'-phosphate terminus of the DNA molecule and (3) the gap in the DNA molecule is sealed when the DNA ligase catalyses displacement of the AMP residue through the attack by the adjacent 3' hydroxyl group of the DNA [76].

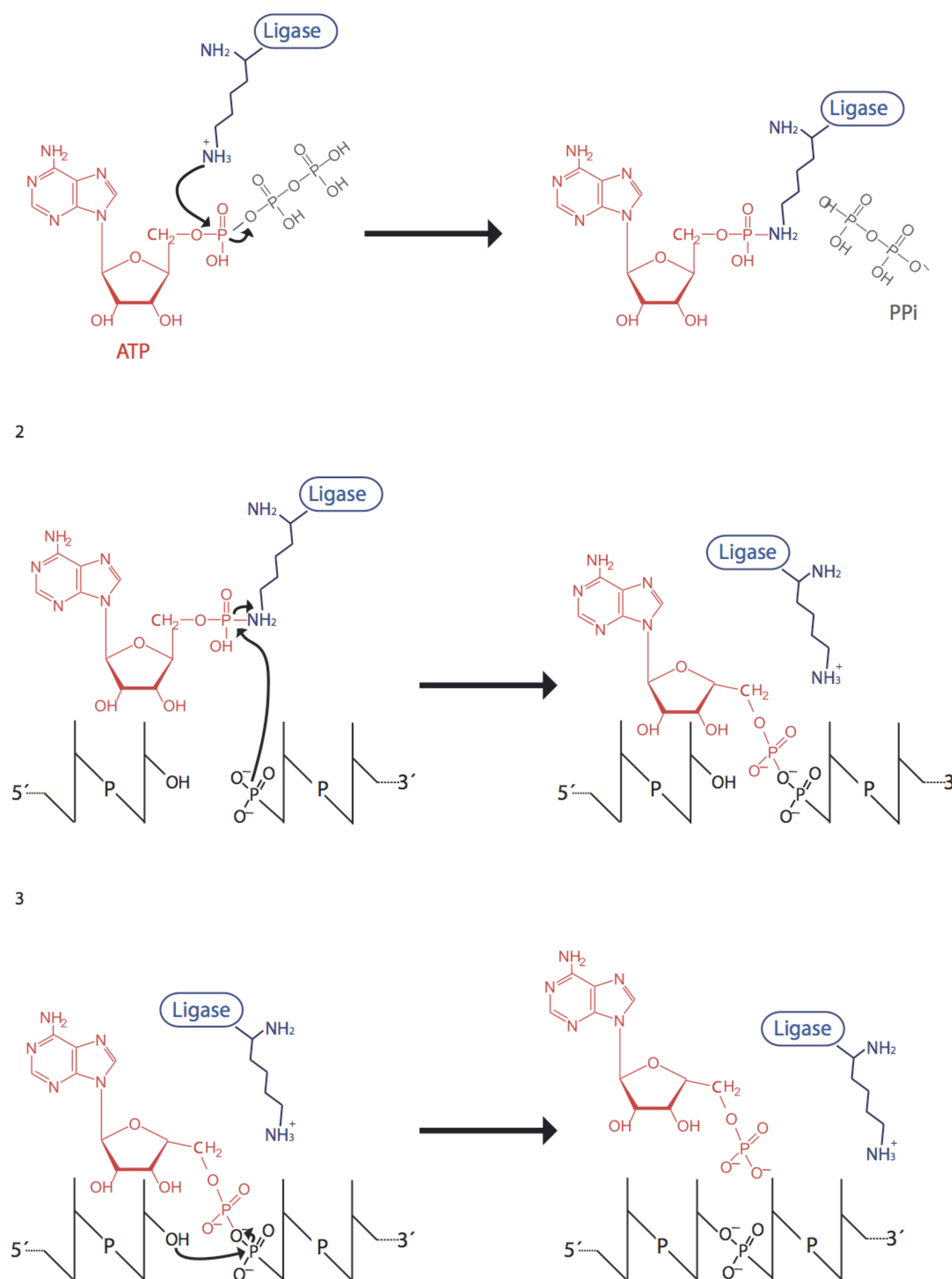

Figure 9 Reaction mechanism for DNA ligases. (1) First a Lys-residue in the ligase enzyme is adenylated using either ATP or NAD+ as cofactor (illustrated here with ATP), making an activated state of the enzyme. (2) The AMP-group is transferred to the 5´-phosphorylated terminus to generate adenylated DNA. (3) Further the formation of the phosphodiester bond is made when the 3´-OH attacks the 5´-phosphorylated DNA end while releasing AMP and covalently joining the DNA strands.

The AMP donor differs and divides DNA ligases into two families: the highly conserved NAD-dependent enzymes, which are found in all bacterial genomes, while some species have an additional ATP-dependent DNA ligase [77]. These classes are structurally different, but the catalytic core is conserved consisting of an oligonucleotide/oligosaccharide binding (OB)-fold domain that contains conserved residues assisting in step 1 of the adenylation reaction [77], and a nucleotidyltransferase (NTase) that houses six conserved motifs and comprises the adenylation domain (AD) where the AMP cofactor is covalently bound [78]. NAD-dependent DNA ligases also possess a unique N-terminal Ia domain essential for sealing nicks in DNA and binding to NAD^+ [79].

1.1.3 DNA replication

1.1.3.1 The replication machinery

To avoid errors in the replication of the DNA, it is important for a cell to have a precise and well functioning duplication system. The replication fork assembles many different proteins that are involved in this precise copy mechanism. For replication to take place the DNA duplex is unwound by topoisomerase, and the binding of an initiator protein to the origin of replication leads to an opening of dsDNA. The resulting ssDNA is further bound to single-stranded DNA binding proteins (SSB) with a purpose to keep the complementary DNA strand separated. For the DNA polymerase to be able to perform DNA synthesis a special RNA polymerase named primase initiates the synthesis by adding a primer that generates a duplex DNA segment. The primase makes a complex with the DNA helicase that unwinds the duplex DNA. Together they are called the primosome, and they slide along the ssDNA in the 5´ to 3´ direction unwinding duplex DNA and laying primers every 1-2 kb DNA in the lagging strand. The DNA polymerase III (replicase) associates to the primed segment of DNA by the β-clamp subunit of the polymerase around the primed DNA to form a ring-shape that can slide along DNA. It further binds the DNA polymerase III holoenzyme with a purpose to ensure that it stays bound to DNA during replication. Since the polymerase only synthesises new chains in the 5´ to 3´ direction, it moves in a 3´ to 5´ direction on the template strand. This means that the leading strand has the polymerase III only loaded once before the DNA is synthesised continuously in great amounts before dissociated [80]. The lagging strand, on the other hand, is synthesised in shorter discontinued fragments (Okazaki fragments)

in the opposite direction of the formation of the replication fork. As the fork movement exposes a new section of lagging-strand template, a new lagging-strand fragment is begun and proceeds away from the fork until it reaches the preceding fragment (**Figure 10**). The lagging strand consists of fragments of primer segments connected to duplex DNA. The 5´ to 3´-exonuclease activity of DNA polymerase I remove the primer segments leaving a gap, which is ligated by ligase.

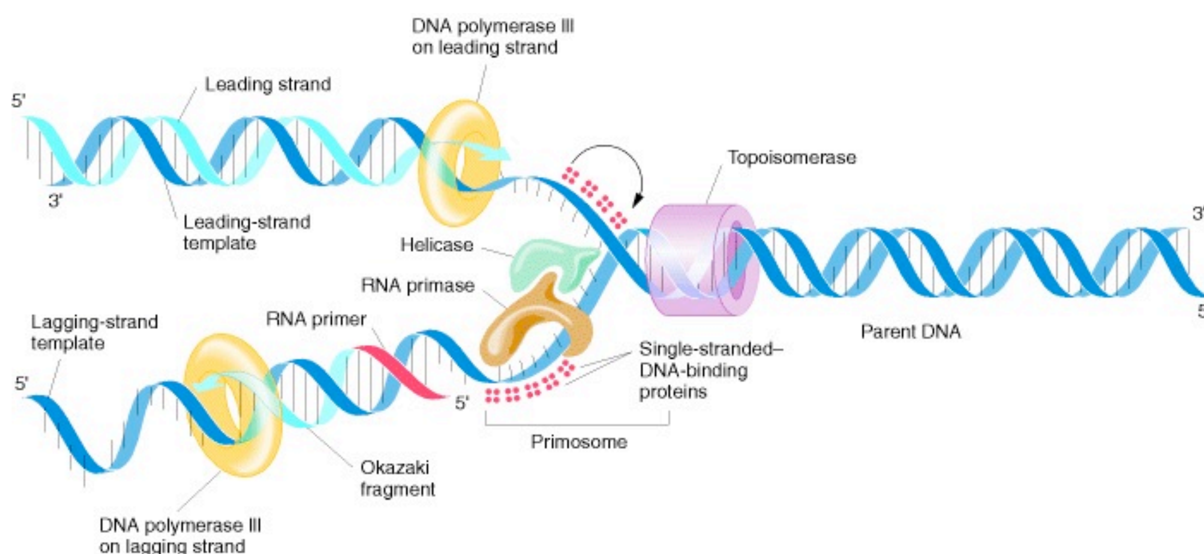

Figure 10 Replication fork. A topoisomerase (purple) unwinds supercoiling making room for replication to take place. The helicase unwinds the dsDNA making room for a RNA/DNA primer (pink) made by the primase where DNA polymerase III binds to initiate the leading-strand synthesis and every Okazaki fragment on the lagging strand. Figure reprinted with permission from [81].

1.1.3.2 Sliding-clamp

The bacterial DNA polymerase III β subunit (β-clamp) is a ring-shaped protein that wraps around DNA, and acts as a docking site for other polymerase subunits linking them to DNA and ensures a more efficient processivity [82]. It also binds a wide variety of other proteins that act on DNA, including DNA ligase, endonucleases and glycosylases (reviewed in [83]). β-clamps exist as closed rings in solution [84], and in order to be loaded onto DNA they need the help of ATP-dependent clamp loaders. Clamp loaders are five-subunit AAA^{+} ATPases [85] where ATP hydrolysis are coupled to conformational changes that enable the clamp loader to open the sliding clamp and place it on DNA [82, 86]. In the case of the catalytic core of *E. coli* DNA polymerase, the replication speed is

increased from approximately 20 nt/s with frequent dissociation [87] to approximately 750 bases per second with a processivity of >50 kb in the presence of the β-clamp [88].

The overall structure of the sliding-clamp is conserved across all domains of life [89, 90]. Although they share no particular sequence similarity, the bacterial β-clamp and proliferating cell nuclear antigen (PCNA) found in eukaryotic and archeae all adopt a ring shaped architecture, where the inner ring is large enough to fit duplex DNA [82, 89-91]. The bacterial β-clamp is a homodimer, where each monomer consists of three domains [89]. The eukaryotic PCNA also has a hexagonal symmetry, but differs by forming a homotrimeric ring [92] (**Figure 11**). The six domains within each enzyme are topologically identical [90]. The sliding clamp protomers are all arranged head-to-tail with an outer layer of 6 β-sheets that supports the 12 α-helices lining the inner surface of the ring (**Figure 11**), giving the ring a distinct front and back referred to as N-terminal and C-terminal face [93]. The C-terminal face has a hydrophobic pocket on the surface of every domain that is conserved among bacterial β-clamp and PCNA [84]. All proteins associated with the sliding clamp are believed to interact through this pocket [93]. There are two structures of sliding clamp bound to DNA available in the protein data bank (*E. coli*, PDB: 3BEP [93]) and *Saccharomyces cerevisiae* (PDB: 3K4X [84]). The most profound discoveries were that in *E. coli* the DNA tilts when bound to the sliding clamp, causing the DNA to make contact with exposed residues Gln149 and Arg24 located in the C-terminal face of the enzyme [93]. These residues are found to be important for DNA replication by mutational studies. The positively charged residues in the inner ring of the PCNA in *S. cerevisiae* help the clamp loader when PCNA is placed onto DNA [84]. But more studies in this field must be performed to fully understand the sliding clamp - DNA interaction.

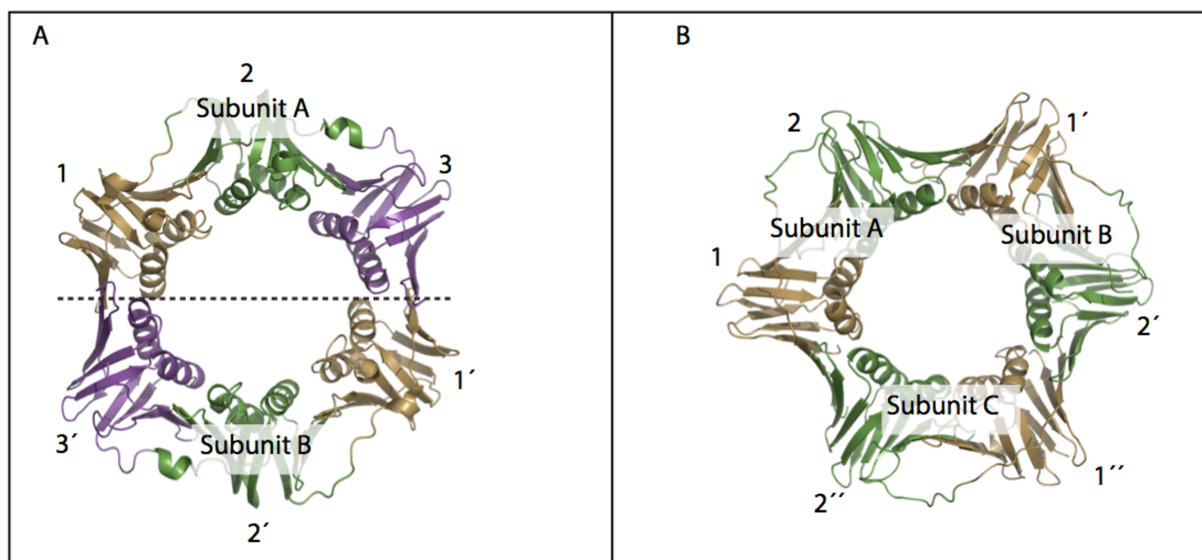

Figure 11 (A) β-clamp from *Escherichia coli* (PDB: 2POL) [89] and (B) PCNA from Homo sapiens (PDB: 1AXC) [94] adopt a ring shaped architecture, where the inner ring is large enough to fit duplex DNA. The bacterial β-clamp is a homodimer, where each monomer (subunit A and B) consists of three domains (1, 2, 3 and 1´, 2´, 3´, respectively), while the eukaryotic PCNA forms a homotrimeric ring, where each subunit (subunit A, B and C) consists of 2 domains (1, 2, 1´, 2´ and 1´´, 2´´, respectively). Figures are made in Pymol (The PyMOL Molecular Graphics System, Version 1.4 Schrödinger, LLC).

1.2 *Aliivibrio salmonicida*

Aliivibrio salmonicida (A. salmonicida) is a psychrophilic, curved gram-negative rod bacterium (**Figure 12A**) with an optimal growth temperature around 12-16°C in liquid culture (**Figure 12B)** [95, 96]. The bacterium is the causative agent of cold-water vibriosis (also called hemorrhagic syndrome or Hitra disease) in Atlantic cod and salmon [97-100]. Vaccines against the disease have been developed but the molecular mechanisms of host invasion and specificity are unknown. The genome of *A. salmonicida* has been sequenced [101] and expression profiling and structural biology projects have been initiated in order to possibly identify the virulence factors of this organism.

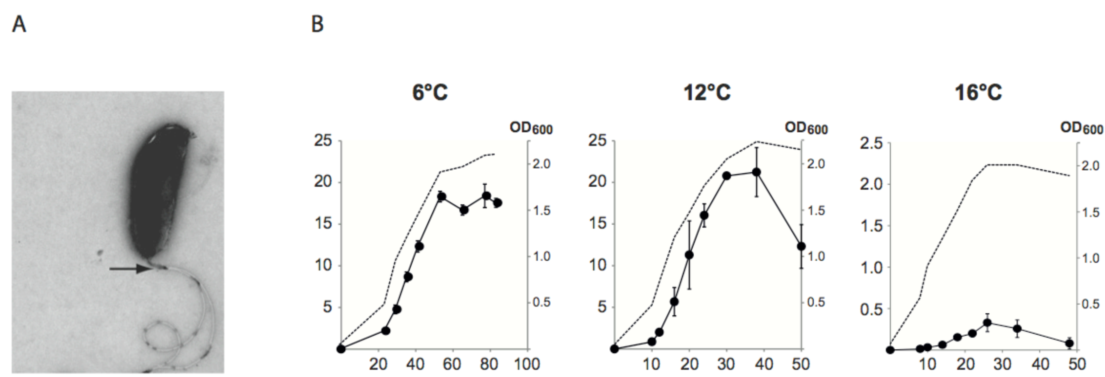

Figure 12 (A) Transmission electron microscopy image of the psychrophilic *A. salmonicida* and (B) growth curve (dotted lines) of *A. salmonicida* at three different temperatures *A. salmonicida* picture and graphical views from [102] and [103], respectively with permission. The uniform line is N-acyl-homoserine lactones produced by *A. salmonicida* and should be ignored in this context.

A vast amount of the Earth consists of cold environments, including deep see waters, the Polar regions, glacier, mountain regions and permafrost. Organisms found in these areas have to adapt to the surrounding environment, and they are dependent on possessing enzymes with an acceptable activity and stability in order to maintain the functionality of their cellular machinery. Enzymes from these organisms are often referred to as cold-adapted enzymes [104]. Cold-adapted enzymes are represented mostly in bacteria [105-109], archeae [110], algae [111], and yeast [112]. But there have also been cases of characterised enzymes from plants and animals [113]. An enzymatic reaction consists of three main phases: i) The enzyme recognises and binds to the substrate, ii) the substrate induces conformational changes upon the enzyme interaction, leading to product formation, and iii) release of the product. Each of these phases involves weak interactions sensitive to temperature changes [114].

Temperature is one of the most important factors for the activity of enzymes, and the Q_{10}-value tells us when lowering the temperature by 10°C it will induce a decrease in enzyme activity by two to four fold [115], and 20-80-fold if the temperature is decreased from 37°C to zero. It is suggested that cold-adapted enzymes have an increase in flexibility in parts of the molecular structure to compensate for their exposure to cold surroundings [114, 116]. Cold-adapted proteins have been found to have higher catalytic efficiency (k_{cat}/K_M) at low temperatures to withstand this strong temperature dependence on the enzyme activity [104, 117, 118]. Three enzymes studied from *A. salmonicida*, uracil DNA N-glycosylase (*As*UNG) [119], catalase (*As*Cat)[106] and

endonuclease I (*As*EndA) [109], showed higher k_{cat}/K_M than their mesophilic homologs. The higher catalytic efficiency in *As*UNG compared to its mesophilic counterpart *Vibrio cholerae* UNG is explained by the lower K_M-value that indicates that it possesses a higher affinity for its substrate. This lower K_M is unusual for cold-adapted enzymes [115] but it has also been observed for other cold-adapted enzymes like *As*Cat [106], UNG from Atlantic cod (cUNG) [120] and for the anionic salmon trypsin from Atlantic salmon (*Salmo salar*) [68, 121].

The effect of temperature on enzymatic reactions can be described by a general rate equation (**Eq. 1**) derived from the Arrhenius equation [115, 117]:

$$k_{cat} = \kappa\,({}^{k_B T}/_{h})\, e^{-\Delta G\#/_{RT}} \qquad \text{(Eq. 1)}$$

where κ is the transmission coefficient generally close to 1, k_{cat} is the enzyme reaction rate that corresponds to the maximum number of substrate molecules converted to product per active site per unit of time, κ is the transmission coefficient generally close to 1, k_B is the Bolzmann constant (1.38×10^{-23} J K^{-1}), h is the Planck constant (6.63×10^{-34} J s), T is the absolute temperature, $\Delta G^{\#}$ is the energy barrier that needs to be overcome by the ground state of the enzyme-substrate complex to react, and R is the universal gas constant (8.31 J K^{-1} mol^{-1}). From the equation we see that an increase in temperature or a decrease in $\Delta G^{\#}$ will give higher reaction rates indicating that in order to increase the reaction rate at low temperatures, cold adapted enzymes can reduce the $\Delta G^{\#}$ of the enzymatic reaction. And a reduction in $\Delta G^{\#}$ makes catalysis more easy at low temperatures, thus the decrease in ΔG# in an enzymatic reaction of psychrophilic enzymes is the main adaptation to low temperature [122]. $\Delta G^{\#}$ is dependent on both enthalpy ($\Delta H^{\#}$) and entropy ($\Delta S^{\#}$) of activation (**Eq. 2**):

$$\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#} \qquad \text{(Eq. 2)}$$

The main explanation for a reduced $\Delta G^{\#}$ in cold-adapted enzymes lies in the lower $\Delta H^{\#}$-value of the enzyme, which indicates that a reduced number of enthalpy related interactions have to be broken during transition-state formation [113] and that the catalytic rate is thus less dependent on temperature (**Eq. 1**).

Previous findings in cold-adapted enzymes have shown higher negative values of $\Delta S^{\#}$

and suggest that the ground state enzyme-substrate complex have a greater degree of disorder than their mesophilic counterparts (reviewed in Feller *et al.* 2013 [123]). It is universal for all cold adapted enzymes studied so far that $\Delta H^{\#}$ and $\Delta S^{\#}$ values are lower than in their mesophilic homologous [104, 115], including in *As*End*A* [109].

The active sites of homologous enzymes thriving at different temperature spectra are very similar, indicating that the catalytic mechanism and reaction pathway are more or less identical [115]. The means of adapting to cold environment must therefore lie outside of the active sites [124]. Many cold-adapted enzymes have been characterised, and over 30 structures have been solved [118], giving us a better insight into important features for cold-active enzymes to in fact be adapted to the cold.

It is important to note that not all cold-adapted enzymes possess all the features listed below, but use some to achieve efficient catalysis at low temperatures. Cold-adapted enzymes have shown to have longer surface loops but with a reduced number of Pro residues in loop regions and an increased number of Gly residues [123]. Met residues are long with a high degree of freedom that can increase the flexibility locally in an enzyme seeing it has no charge or dipole interaction [104]. Hydrogen-bonds and salt bridges stabilise the protein structure, and Arg is believed to stabilise the protein structure by its ability to form salt bridges and a large number of hydrogen bonds [125] compared to Lys. Therefore, a lower relative Arg content [Arg/(Arg-Lys)] are also seen in some cold-adapted enzymes [126]. This is found in the study of *As*Cat where the numbers of Arg residues is 26 compared to 31 in the mesophilic homologue [106], and numbers of salt-bridges are reduced [105].

Other features that could explain cold-adapted features are the lack of aromatic and ionic interactions [123] that are important for stabilising the protein structure. The molecular surface seems to be important for cold adaptation, and enzymes from cold climate tend to have a higher proportion of hydrophobic residues at the surface [113]. The hydrophobic residues induce clusters of water molecules, which gives a decrease in entropy ($\Delta S^{\#}$) thus destabilising the protein structure in solution [123].

Even though every psychrophilic enzyme has their own unique features to explain their cold-adaptation, most share the property of heat-labile activity. That said, there are several cold-adapted enzymes characterised that show high thermostability as *e.g.*

isocitrate dehydrogenase from *Desulfotalea psychrophila* [127], Aspartase from *Cytophaga sp.* KUC-1 [128], alcohol dehydrogenase *Flavobacterium frigidimaris* KUC-1 [129]) indicating that there can be a significant difference between the stability of the active site and the stability of the protein structure.

1.3 *Deinococcus radiodurans*

Deinococcus radiodurans (*D. radiodurans*) is a gram-positive bacterium with a pink-orange colour (**Figure 13A**), first isolated from canned meat [130], but no one really knows the microbe's natural habitat. It exhibits outstanding resistance to ionising radiation (IR), ultraviolet-light (UV-light) and desiccation [131, 132] (**Figure 13B**). Whereas most organisms cannot survive doses above 50 Gray [133-135], *D. radiodurans* tolerates doses ranging from 5,000 to 30,000 Gray [136] (**Figure 13C**). Such massive radiation doses are estimated to induce several hundred DSBs, thousands of single-strand gaps and about one thousand sites of DNA base damage per chromosome [137, 138]. The annotated sequence of the *D. radiodurans* genome was published in 1999 [139] and allowed a detailed analysis of the genomic composition of this organism. The resistance mechanism of *D. radiodurans* is not known, but initial investigations have suggested that it is complex and possibly determined by a combination of factors such as genome packing, cell structure and a highly efficient DNA repair machinery [139-142]. In the later years the focus has changed, and together with an efficient DNA repair system it is also believed that the cell has a very efficient protein-damage defence system (discussed in [142, 143].

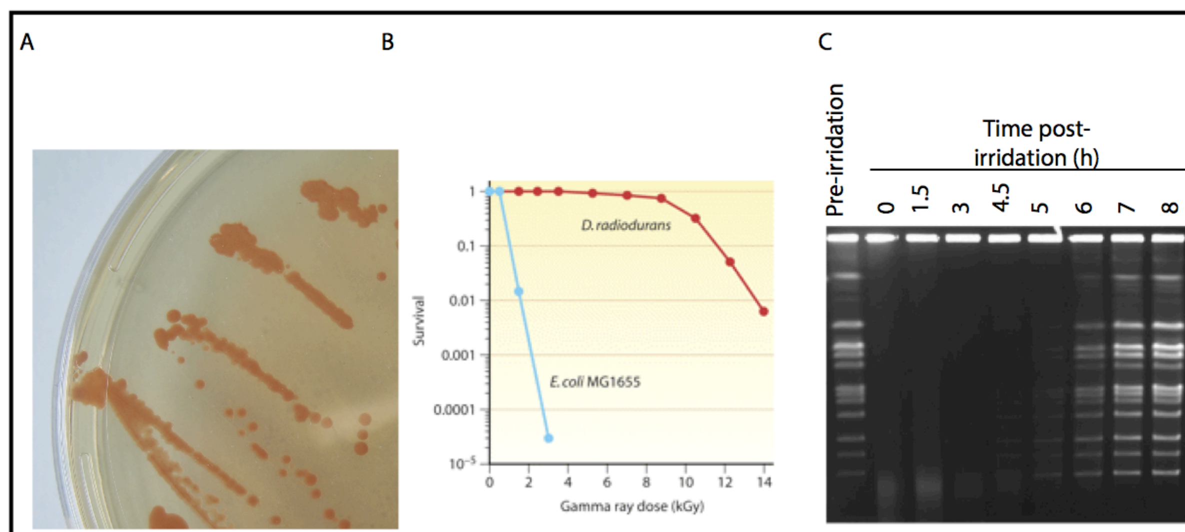

Figure 13 (A) *D. radiodurans* grown in tryptone-glucose-yeast extract (TGY)-medium illustrating its characteristic pink-orange colour due to carotenoid pigment (photo by Joanna Timmins, used with permission). (B) Extreme resistance of *D. radiodurans* to gamma-rays compared to *E. coli*, (C) Kinetics of DNA double-strand break repair in *D. radiodurans* cells after 14 kGray of gamma rays. Samples of unirradiated and irradiated wild-type cells were taken to prepare DNA plugs, which were digested with the restriction endonuclease *Not*I, generating 12 visible fragments. Lane "0" shows the *Not*I restriction pattern of DNA from irradiated cells immediately after irradiation, and subsequent lanes show the *Not*I restriction patterns of DNA from cells at different time points after irradiation, expressed in hours. Figures B and C reused with permission [143].

1.3.1 Radiation resistance of *D. radiodurans*

D. radiodurans is remarkable amongst all species studied to date by its unusual ability to repair hundreds of radiation induced DNA DSBs where normal species can only repair a dozen [144, 145]. Possible explanations for *D. radiodurans´* rapid repair of damage could be that its enzymes can work at an incredible high speed and/or that the concentrations of repair proteins are enhanced compared to radiation sensitive organisms, but studies performed on the DSB repair enzymes *Rec*A and *Pol*A showed no evidence that they exists in higher concentration in *D. radiodurans* than in other bacteria [145-147]. Surprisingly, DSB caused by radiation were found at equal amounts among radiation-resistant and -sensitive organisms examined [145, 147].

In later years there have been studies on the amount of oxidised proteins in cells exposed to radiation, and it has been found that oxidation to proteins negatively correlates to survival [143]. This led to the proposal that it is the proteins that are the

targets for damage during radiation, and if the organism is able to protect the DNA repair proteins they can perform DNA repair as normal. This proposal would also explain why radiation-sensitive bacteria are not able to repair lesions in DNA, since their repair proteins would be inactivated. In 1976, Leibowitz *et al.* [148] found that *D. radiodurans* had higher concentration of Mn^{2+} than the radiation-sensitive *Micrococcus luteus*. This led to further studies in which it was found that several radiation-resistant bacteria had a higher Mn^{2+}/Fe^{2+}-ratio than radiation-sensitive bacteria [149], and researchers hypothesised that the higher accumulation of Mn^{2+} in cells can prevent oxidation in proteins by preventing the formation of iron-dependent ROS as discussed in [145]. Even though *D. radiodurans* is a bacterial super-hero, it must repair the damages that occur due to oxidation in DNA and proteins. Since one consequence of unrepaired damages is cell death [150], efficient DNA repair mechanisms is critical.

The importance of the DNA machinery for the radiation resistance has also been subject of studies over the last ten years. Regarding the BER pathway it has been shown that *D. radiodurans* is unique in having elevated numbers of DNA glycosylases [151], with 11 identified so far [143]. Amongst them, UNG (dr0689) has been proposed to be the major contributor of the removal of misincorporated uracil bases [152], and have been shown to possess a four times higher catalytic efficiency than human UNG [153]. The mismatch-specific uracil-DNA glycosylase (MUG) was found to possess wide substrate specificity with the ability to remove uracil from both U:G and U:A mismatches in dsDNA as well as uracil in single-stranded DNA. It was proposed that the broader substrate specificity could improve the resistance mechanism of the organism against radiation and desiccation [154]. *D. radiodurans* is also unusual in possessing three genes encoding the oxidative stress related DNA glycosylase Endonuclease III (EndoIII-1, EndoIII-2, and EndoIII-3) [155], and two genes encoding the alkylation damage repair enzyme 3-methyladenine DNA glycosylase II (AlkA-1 and AlkA-2). The crystal structure determination and characterisation of AlkA-2 showed an unusual crystal structure of the enzyme, with the absence of a domain typically seen for this enzyme, and broader substrate specificity as in the case of MUG [156]. The importance of having three EndoIII enzymes is still not known, however it indicates an improved ability to repair oxidation damaged DNA, damages which are highly increased upon exposure of bacteria to radiation and desiccation [146].

The RecBCD complex (and its functional homolog AddAB) is considered the main recombinational DSB repair pathway in bacteria, however in *D. radiodurans* the major components of this pathway is not present [157]. Instead *D. radiodurans* possesses homologs of the key components of the alternative RecF pathway, and knock out studies of RecF, RecO, RecR and RecA has shown that all these proteins are required for massive DNA synthesis during DSB repair in *D. radiodurans*, thus suggesting that this is the main recombinational repair pathway in this organism [158]. In addition to RecF, RecO and RecR the RecF pathway contains the 3´-5´ helicase RecQ and the 5´- 3´nuclease RecJ [159, 160], and crystal structures of the three former proteins and the RecOR complex have been reported confirming the existence of a functional RecFOR pathway in *D. radiodurans* [159, 161-165]. Interestingly, it has also been shown that the genome could be slowly regenerated in the absence of RecA, RecF, RecO or RecR, suggesting that an alternate DSB repair pathway exist in *D. radiodurans* [158]. The importance of RecF being the main recombinational repair pathway and the presence of a possible alternative pathway for the radiation resistance of *D. radiodurans* is still not known.

D. radiodurans has two UV repair pathways (UvrABC and UV damage endonuclease, UVDE). The former consists of four Uvr proteins: UvrA, UvrB, UvrC, and DNA helicase II, UvrD. UvrA acts as a dimer with UvrB and together they are responsible for DNA damage recognition. The crystal structures for both UvrA2 [166] and UvrD [167] have been solved. In UvrA2, it is suggested that the insertion domain together with the C-terminal zinc finger are critical for damage recognition [166]. UvrD is as its homologs an active DNA stimulated ATPase with ATP-dependent translocase and helicase activities, but differs by its ability to unwind duplexed DNA in both the 3´-5´and 5´-3´directions [167]. Recently it was also discovered that UvrD might be involved in DNA double-strand-break repair in *D. radiodurans* [158], thus indicating multiple roles for this enzyme in DNA repair in this organism

2. Background and aims of the study

This thesis is based on work, which was performed as part of a project funded by The Research Council of Norway (RCN) through the functional genomics (FUGE) programme to study protein complexes in the Base Excision Repair (BER) pathway in the two extremophiles *Aliivibrio salmonicida* and *Deinocococcus radiodurans*. These two model organisms were chosen based on ongoing functional and structural genomics projects at UiT, the Arctic University of Norway and in our collaborators´ laboratory at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France.

In this project, the main objective was to characterise at a biochemical and structural level the downstream processing BER apparatus (AP-endonuclease, DNA polymerases and DNA ligases) and parts of the replication machinery of *A. salmonicida* and *D. radiodurans* in order to study their function related to their extremophilic phenotypes. In addition structure/function studies of MutT from *A. salmonicida* and *V. cholerae* were included.

One of the bottlenecks in studies of recombinant enzymes for structural analysis is to obtain high yields of soluble protein, thus the project started by cloning and testing the expression of 10 selected targets (**Table 2**) in addition to MutT from *A. salmonicida* and *V. cholerae*.

Table 2 Overview of down-stream enzymes from *A. salmonicida* and *D. radiodurans*

Organism	Proteins	Entry
Deinococcus radiodurans	Exonuclease III (AP-endonuclease) – *Dr*ExoIII	DR_0354
	DNA polymerase I – *Dr*PolI	DR_1707
	DNA ligase (NAD dependent) – *Dr*LigA	DR_2069
	DNA polymerase III β-subunit – *Dr*β-clamp	DR_0001
Aliivibrio salmonicida	Endonuclease IV (AP-endonuclease) – *As*EndoIV	VSAL_I2552
	Exonuclease III (AP-endonuclease) – *As*ExoIII	VSAL_I1962
	DNA polymerase I – *As*PolI	VSAL_I0227
	DNA ligase (NAD dependent) – *As*LigA	VSAL_I2353
	DNA ligase (ATP dependent) – *As*LigB	VSAL_I1366
	DNA polymerase III β-subunit – *As*β-clamp	VSAL_I0010

Based on the results from the initial cloning and expression tests the following aims for the thesis were determined:

1. Perform comparative biochemical and structural study of MutT from *Aliivibrio salmonicida* and *Vibrio cholerae* in order to determine molecular determinants for cold adapted properties of MutT from *A. salmonicida*.
2. Determine the crystal structure of *D. radiodurans* β-clamp (*Dr*β-clamp), in order to study molecular determinants for efficient DNA replication in *D. radiodurans*.
3. Perform biochemical characterisation of Exonuclease III from *Deinococcus radiodurans* (*Dr*ExoIII) in order to study its importance for DNA processing glycosylases and of AP-sites in *D. radiodurans*.

3. Summary of results

3.1 Protein production

The genes encoding the identified downstream DNA repair enzymes from *D. radiodurans* and *A. salmonicida* were all cloned as constructs with nucleotides encoding an N-terminal Glutathione S-transferase (GST) tag and a TEV protease cleavage site and were screened for expression in three different *E. coli* strains (BL21 (DE3), BL21 star (DE3) and BL21 star (DE3) pLysS) at three different temperatures (15, 20, 30°C). The expressed constructs were further expressed in large scale and purified. An overview of expressed targets are summarised in **Table 3**.

Table 3 Overview of results from cloning and expression tests of *D. radiodurans* and *A. salmonicida* targets

Organism	Proteins	Strains	Temperature	Large scale expression	Purification
Deinococcus radiodurans	Exonuclease III *Dr*ExoIII	No expression			
	DNA polymerase I *Dr*PolI	BL21 star (DE3)	20 and 30°C	X	Low yield
	DNA ligase (NAD^+ dependent) *Dr*LigA	BL21 star (DE3)	20°C	No growth during large scale expression	-
	DNA polymerase III β subunit *Dr*β-clamp	No expression			
Aliivibrio salmonicida	Endonuclease IV *As*EndoIV	BL21 (DE3)	20°C	X	DNA contamination and protein precipitation
	Exonuclease II *As*ExoIII	BL21 (DE3)	20°C	X	DNA contamination
	DNA polymerase I *As*PolI	BL21 (DE3) pLysS	30°C	X	Low yield
	DNA ligase (NAD^+ dependent) AsLigA	BL21 star (DE3) pLysS	30°C	X	>90% insoluble
	DNA ligase (ATP dependent) *As*LigB	BL21 star (DE3)	20°C	No growth during large scale expression	-
	DNA polymerase III β subunit *As*β-clamp	BL21 star (DE3)	30°C	X	>90% insoluble

The GST tag was chosen in order to facilitate the analysis of protein-protein complexes, which were the ultimate goal of the RCN funded project. In general we observed from the expression test that the *A. salmonicida* enzymes had poor solubility or high amounts of DNA contaminations and the *D. radiodurans* enzymes showed low or no expression. Closer inspections of some of the *D. radiodurans* genes revealed several errors in the annotation, potentially explaining the low expression levels of these enzymes [168]. The sequence analysis of *Dr*β-clamp revealed that the initially cloned gene contained a deletion of cytosine 1039 compared to the published genomic sequence of *D. radiodurans* strain R1 [139]. This leads to a frame shift and an earlier stop codon producing a shorter version of the gene, which encodes a 362 amino acid protein more similar both in length and in sequence to other known β-clamps. Looking closer into already solved structures of sliding clamps and proliferating cell nuclear antigen (PCNA) it also looked like an N-terminal tag could potentially prevent dimerisation of the protein, thus it was decided to clone both the long and the short version of the gene with nucleotides encoding a His_7-tag attached to the C-terminus of the protein. The His-tag (and TEV cleavage site) was now chosen based on our earlier good experience in using this tag in expression of recombinant enzymes. Only the short version of *Dr*β-clamp could be successfully expressed.

Similarly, a closer analysis of the annotated gene of *Dr*ExoIII revealed that it has an N-terminal extension of about 30 amino acids compared to its bacterial homologues. Also, residue 23 is a Met amino acid and could be a alternative start position for gene expression, thus it was decided to prepare a 22 amino acid N-terminally truncated version of *Dr*ExoIII (*Dr*ExoIIIΔ22). Both *Dr*ExoIIIΔ22 and *Dr*β-clamp (short) were soluble and expressed in large amounts and used in further studies in this project.

The expression of the MutT enzymes from *A. salmonicida* and *V. cholerae* had been analysed earlier, and were also included in this work.

3.2 Manuscript I

MutT from the fish pathogen *Aliivibrio salmonicida* is a cold active nucleotide pool sanitization enzyme with an unexpected high thermostability.

Kjersti Lian, Hanna-Kirsti S. Leiros and Elin Moe.

FEBS Open Bio, February 3, 2015. 5:107-16. *doi:10.1016/j.fob.2015.01.006*

In this paper we have performed a comparative study of MutT from *A. salmonicida* and *Vibrio cholerae* in order to determine how this enzyme prevents oxidised nucleotides from being incorporated into the fish pathogens' genome in an efficient way in cold environments. We determined the crystal structure of *V. cholerae* MutT, generated a homology model of *A. salmonicida* MutT and performed biochemical assays and stability measurements of both proteins. Our findings show that *As*MutT possesses cold adapted properties with a higher catalytic efficiency than *Vc*MutT mainly caused by higher k_{cat} values, lower K_M, and lower activation energy (E_a) due to lower activation enthalpy ($\Delta H^{\#}$). However *As*MutT exhibits an unexpected higher overall stability than *Vc*MutT with a T_m of 57.80 ± 0.09 °C compared to 54.70 ± 0.22 °C for that latter. By generating homology models of *As*MutT and comparing them to the crystal structure of *Vc*MutT, we were able to identify structural determinants, which may explain our biochemical and biophysical data.

3.3 Manuscript II

Crystal structure of the DNA polymerase III β subunit (β-clamp) from the extremophile *Deinococcus radiodurans*.

Laila Niiranen, **Kjersti Lian**, Kenneth A. Johnson and Elin Moe.

BMC Structural Biology, 2015, 15:5, doi:10.1186/s12900-015-0032-6

In this study we studied the crystal structure of the DNA polymerase III β subunit from *Deinococcus radiodurans*. We found that the gene encoding *Dr*β-clamp was wrongly annotated in the genome database, encoding a protein of 393 instead of 362 amino acids. The short protein was successfully expressed, purified and used for crystallisation purposes in complex with Cy5-labeled DNA. The structure was determined to 2.0 Å resolution and showed typical dimer ring-shaped bacterial β-clamp formed of two monomers. Each monomer had three domains with identical topology, unfortunately with no DNA visible in the observed electron density. The calculated electrostatic surface potential revealed a negatively charged outer surface while the inner surface and the dimer interface had a more neutral charge distribution, indicating a less tight binding and thus a more efficient sliding on DNA compared to other structurally determined bacterial β-clamps.

3.4 Manuscript III

Biochemical characterisation of Exonuclease III from the extreme radiation and desiccation resistant bacterium *Deinococcus radiodurans*

Kjersti Lian, Joanna Timmins and Elin Moe.

Manuscript, 2015

Here we have analysed the biochemical properties of the 22 nt truncated ExoIII from the extremely radiation and desiccation resistant bacterium *Deinococcus radiodurans* (*Dr*ExoIIIΔ22) in order to determine its role in the highly efficient DNA repair machinery of this unusual phenotype. The results show that *Dr*ExoIIIΔ22 possesses AP-endonuclease, 3'-5' exonuclease activity and 3'diesterase activity on dsDNA, and that the former is dependent on the presence of magnesium and that exonuclease activity is salt sensitive in the presence of $MgCl_2$. Homology modelling indicates that *Dr*ExoIIIΔ22 adopts a four-layered α/β-sandwich motif as seen in other members of the *Xth*-family of AP-endonucleases, and that all the catalytic residues known to participate in AP-site recognition are conserved. The homology model of *Dr*ExoIIIΔ22-Mg^{2+} reveals that Glu69 and Asn43 are placed in positions enabling Mg^{2+} binding and moves upon binding to substrate DNA, explaining the Mg^{2+} dependence of the AP-endonuclease activity of this enzyme as also observed for APE1 and *Ec*ExoIII. The enzyme contains two tryptophan residues, instead of only one as observed in APE1 and *Ec*ExoIII, in positions, which have been shown to be important for AP-site recognition, indicating an efficient means of substrate recognition of *Dr*ExoIIIΔ22. Finally, a visualisation of the electrostatic surface potential of the apo-structure homology model shows a less positively charged active site pocket of *Dr*ExoIIIΔ22 compared to APE1, possibly explaining the observed salt sensitivity of the 3'-5' exonuclease activity in presence of $MgCl_2$. We hypothesise that this salt sensitivity might be of biological importance for *D. radiodurans* upon desiccation and a following increase in the intracellular salt concentration, pressing the enzyme specificity towards AP-endonuclease activity, which under these conditions will be more important for the maintenance of the genome stability.

4. Discussion

4.1 Cold adapted properties of *AsMutT*

Since there are no common structural features found for all enzymes from cold-adapted organisms, characterisation and structural determination of individual enzymes are important to explore their potential and differences. Cold-adapted enzymes have been suggested to have a more flexible structure since catalysis is more challenging for enzymes working at low temperature due to slower mobility, lower solubility of substrates and slower reaction rates.

Structural explanations for cold-active properties

By performing differential scanning calorimetry [137] and by activity assay measurements on MutT from both *A. salmonicida* and *V. cholerae* we have found *As*MutT to be a cold-adapted enzyme. Enzyme assays at different temperatures revealed that *As*MutT possesses a lower activation energy (E_a) compared to *Vc*MutT, which allows *As*MutT to perform catalysis more efficiently at low temperatures. Knowing that the main explanation for the reduced E_a lies in the reduced enthalpy ($\Delta H^{\#}$) of the enzyme (**Eq. 3**), and calculations having shown that *As*MutT had lower $\Delta H^{\#}$ than *Vc*MutT, we tried to find an explanation for our observed difference in $\Delta H^{\#}$.

$$\Delta H^{\#} = E_a - RT \qquad \text{(Eq. 3)}$$

All of the interactions between residues in the substrate binding pocket and the substrate are the same in *As*MutT and *Vc*MutT (Figure 4C and 4D, **manuscript I**), indicating that other parts of the enzyme most likely account for the observed enthalpy difference. In a study on cold-adapted anionic salmon trypsin (AST) compared to its mesophilic counterpart Bovine trypsin [169], it was suggested that the different enzyme-water interactions could explain reduced $\Delta H^{\#}$ by fewer H-bonds. On the surface of *As*MutT and *Vc*MutT we found that there are many residue substitutions. *As*MutT has more charged residues (highest amount of negative residues) on its surface that could prefer to interact with bulk solvents, while *Vc*MutT has polar residues that may interact in extensive H-bonding networks back to the protein surface. An excess of negatively charged residues at the surface of the protein has been seen for other psychrophilic enzymes and is thought to be related to improve interactions with the solvent, which

could be of prime importance in the need of flexibility near zero degrees [123]. To be sure of this hypothesis, we should ideally have high-resolution crystal structures of both enzymes, which may enable us to see this possible protein surface to water interactions.

As stated earlier, cold-adapted enzymes usually show a higher catalytic efficiency (k_{cat}/K_M) than their mesophilic homologues at low temperatures in order to withstand the strong temperature dependence of the reaction rate [104, 117, 118]. Ideally enzymes should optimise both k_{cat} and K_M to be functionally adapted to the cold, but in most cases psychrophilic enzymes optimise their catalytic efficiency by improving their reaction rate value at the expense of K_M [113]. The higher catalytic efficiency for *As*MutT is mainly caused by high k_{cat} values, but also slightly lower K_M values at most of the temperatures tested (18, 25, 32 and 37°C).

The lower K_M value indicates that *As*MutT possesses a higher affinity for its substrate than *Vc*MutT. Although unusual for cold adapted enzymes it has also been seen for UNG from both cod and *A. salmonicida* where the negatively charged DNA substrate binds strongly to a positively charged active site [119, 170]. To try to explain why *As*MutT has lower K_M-values compared to *Vc*MutT, we compared the modelled structures of both enzymes in closed conformation bound to 8-oxodGMP using *Ec*MutT bound to 8-oxodGMP and Mn^{2+} as template (PDB: 3A6U) (Figure 4E and 4F, **manuscript I**). This is believed to be the active substrate bound form, where the enzyme is closing around its substrate during catalysis. We found that although the overall charges for *As*MutT and *Vc*MutT are almost identical (-7 and -8, respectively), they have a slightly altered charge distribution. On the tip of the loop between β2 and β3 and in β5 prior to the loop between β5 and β6, *As*MutT have a unique Lys27 (His27 in *Vc*MutT) and His74 (Phe74 in VcMutT). These loops change conformation upon substrate binding in *E. coli* MutT [42], and we also see this in our models where it moves closer to the substrate-binding site. In the Nudix box motif ($GX_5EX_7REUXEEXGU$) localised in the active site of the enzyme, *As*MutT has an Asn56 compared to the negatively charged Glu56 in *Vc*MutT. Seeing that the substrate for MutT has a negatively charged triphosphate tail, the negative Glu56 in *Vc*MutT would potentially give lower binding affinity while the two extra positive residues in *As*MutT and their possible contribution to a difference in local electrostatic potential can be essential for binding affinity, as well as orienting and attracting the substrate prior to contact with the enzyme.

The substrate turnover (k_{cat}) was found to be higher for *As*MutT in all temperatures measured compared to *Vc*MutT, which is seen in most psychrophilic enzymes and can be explained by a decrease in activation enthalpy. The k_{cat} values are comparable at their natural physiological temperatures, 12°C for *A. salmonicida* and 37°C for *V. cholerae*, meaning they exhibit the same magnitude of thermal motions.

Structural elements explaining unexpected high stability

The differential scanning calorimetry determined *Vc*MutT to have a T_m at 54.70°C, while *As*MutT revealed a high T_m at 57.80°C. The high thermostability for *As*MutT was quite unexpected seeing it has cold-adapted properties as discussed above, and lack thermophile characteristics as *e.g.* large networks of ionic interactions in active-site regions as seen in *Thermotoga maritima* IDH [127]. *As*MutT has a lower content of Arg residues compared to *Vc*MutT (4 and 6, respectively) (Table 3, **manuscript I**). Since Arg has the ability to form multiple salt bridges and H-bonds thus increasing stability, this does not support our findings on higher thermal stability for *As*MutT. On the other hand, it is in good agreement with other cold-adapted proteins that often are characterised by a lower Arg/(Arg + Lys) ratio compared to mesophilic enzymes [104].

A broader global stability has been found in several other cold-adapted enzymes studied so far [127-129]. A closer look at the amino acids composition of the two enzymes revealed that *As*MutT possesses less glycine residues than *Vc*MutT. Two of the additional glycine residues in *Vc*MutT were found within the β-strands β1 (Gly9) and β7 (Gly104). Since both *Vc*MutT and *Ac*MutT have a Glysine in position 103, this provides a local mobility in *Vc*MutT with a cluster of two Gly-residues. Still, the Gly-residues located at the surface are likely to contribute to the reduced thermal stability of *Vc*MutT (Figure 5A, **manuscript I**). The different Gly on the surface of *Vc*MutT are located in the loop between β4 and α1 (Gly44), between β6 and β7 (Gly93) and in the disordered C-terminal (Gly132), where *As*MutT in comparison has the charged Asp44 and His93, and the hydrophobic Leu132, respectively. The latter can contribute to a more stable enzyme since hydrophobic residues can take part in possible extensive H-bonding networks back to the protein surface [169].

*As*MutT also contains one less Met (Met51 in *Vc*MutT) and one extra Pro (Pro107) residue compared to *Vc*MutT, where the former could increase the flexibility locally in

*Vc*MutT. The pyrolidine ring in Pro restricts the residue from having to many conformations and this lower backbone configuration, which reduces the unfolding [171]. *As*MutT Pro107 is located in the first turn of the α-helix η1 (Figure 2, **manuscript I**) where it most likely stabilises the enzyme as seen previously where Proline residues stabilise surface loops, β-turns, the first turn of α-helixes and at the N-cap of α-helixes [172].

Ionic interactions and increased formation of large ionic networks at the protein surface has shown to increase the thermal stability of an enzyme. By performing a comparative structural analysis on *Vc*MutT (PDB: 4V14) and the homology model of *As*MutT made by using *Vc*MutT as template, we analysed the ion pairs and identified some unique long ionic interactions in *As*MutT, including Lys18-Glu110 and Lys90-Glu65 (Figure 5C and 5D, **manuscript I**) that were absent in *Vc*MutT.

It is not easy to hypothesise why *As*MutT has overall higher stability than its mesophilic homologue. Based on the sequence similarity it is safe to say that MutT from *V. cholerae* and *A. salmonicida* have evolved from the same ancestor. But questions to ask are whether the ancestral enzyme lived at high temperature and whether the *As*MutT enzyme of today has adapted for catalysis in the cold? Or have todays´ MutTs evolved from a common ancient cold-active enzyme and *Vc*MutT has had to learn how to function at high temperature? Both are possible explanations. If the former tells the the true story, one explanation for the continues high thermal stability of *As*MutT can be that it does not seem to compromise the catalytic efficiency at low temperatures and there are no enzyme function tradeoffs in its natural low temperature environments. Even though we have not run activity assay at temperatures above 37°C, we speculate that the active site is more heat-labile than the whole protein structure, meaning that the enzyme function of *As*MutT most likely is inactivated by temperature long before the protein structure unfolds [123].

MutT – general discussion

When the *A. salmonicda* bacterium infects its host, the production of ROS is part of the host organism's first line of defense and the bacteria need to have efficient ROS repair systems to withstand this harsh environment. *A. salmonicida* lives and thrives in cold surroundings, with an optimum growth around 12°C where *As*MutT has high catalytic

efficiency. We therefore believe that the MutT protein is important for *A. salmonicida* and its fight against oxygen radicals.

Recent studies on human MutT (MTH1) have shown that there is potential for using MTH1 inhibitors as anticancer drugs [47, 48]. Upon addition of MTH1 inhibitors to cancer cells, the cells were directed to apoptosis and died. Based on these findings and the current knowledge that exists regarding bacterial MutT and their importance for the bacterial resistance to ROS upon infection, we propose that there is a potential for using MutT inhibitors in treatment of bacterial infections both in humans and animals as an alternative to antibiotics.

4.2 Importance of *Dr*β-clamp and *DrExoIII* for radiation resistance in *D. radiodurans*

D. radiodurans is the most radiation-resistant bacteria known to date and it has been intensively studied in order to identify the mechanism for resistant properties. Many models have been suggested and one of them is that the physical shape of the genome allows extremely rapid DNA repair [142]. By having a donut-shaped structure where the genome is tightly packed, even when it is broken, it is easier for the repair mechanisms to mend it. Another proposal is the high amount of manganese in the cells of *D. radiodurans* that counterbalances the harmful effects of oxidation that follow radiation [149]. It is also believed that it is the combination of proteins that helps repair DNA [173]. It could be one of them, or a combination of several. By continuing the study of un-characterised proteins in *D. radiodurans* potentially involved in the resistance mechanism we may contribute to the overall solution for the radiation-resistance, but there is also a possibility to discover new interesting enzyme properties that can expand the use of enzymes into exciting new areas as *e.g.* drug design, analytical methods, pharmaceutical and chemical industry.

In manuscript II, I have presented a structural report on the DNA polymerase III β-subunit, and compared the structure with already studied sliding clamps. Manuscript III focusus on the biochemical characterisation of the enzymatic activities of the AP-endonuclease, *Dr*ExoIII, and presents a homology model of the enzyme. The main conclusions have already been presented in manuscripts II and III. However, a summary of the findings will be briefly presented and discussed here.

4.2.1 *Dr*β-clamp

The β-clamp is required for efficient processivity in bacteria [82], increasing the replication speed for DNA polymerase III by 10-folds. The clamp interacts with a variety of other proteins involved in different aspects of DNA metabolism as *e.g*. MutS, DNA ligase, Polymerase I, II and IV [83, 174, 175], and it has been found that they all bind to a specific region of the protein called hydrophobicity pocket or protein interaction pocket. Based on a sequence alignment with other bacterial β-clamps and the structure of *Dr*β-clamp, this hydrophobicity pocket is also present in *Dr*β-clamp.

*Dr*β-clamp was herein studied and we found that it adapted the well known ring-shaped structure formed of two monomers, although with a more elliptic shape. The β-clamp differed from other sliding clamps as *E. coli* (PDB: 2POL) *Mycobacterium tuberculosis* (PDB: 3P16)[176] and *Thermotoga maritime* (PDB: 1VPK, unpublished) in that the inner side of the ring had a more spread charge forming small positive patches separated by negatively charged areas (Figure 3, **manuscript II**).

We believe this can cause a less tight binding to DNA compared to other sliding clamps, and hypothesise that the clamp is more efficient when sliding on DNA thereby contributing to a more efficient DNA metabolism in *D. radiodurans* upon exposure to high doses of ionising radiation or desiccation. To validate this hypothesis it would have been ideal to determine the crystal structure of a protein-DNA complex to see which residues interact with DNA. Co-crystallisation was performed with *Dr*β-clamp on dsDNA with 4- and 6-nt long single-stranded thymidine overhang (4 T/6 T), labelled with Cy5. The crystals were blue (Figure 5, **paper II**), indicating that the DNA had entered the crystals, however it was not possible to see the DNA in electron density, indicating loose binding to the protein. This is not the first time co-crystallisation with DNA has proven difficult with β-clamp and PCNA as seen for both Georgescu *et. al.* [93] and McNally *et. al.* [84]. For the latter study on the eukaryotic complex structure, the electron density for DNA was weak, which may indicate that the true DNA binding mode is too unspecific and flexible to be well defined using crystallographic methods without cross-linking. In the former study of an *Ec*β-clamp-DNA complex, they found the 4 T-DNA bound inside the ring but oriented in the opposite direction from what was expected. They also identified that the DNA was not interacting in the same molecule as normally seen, but

between the 5′ end of the DNA and the hydrophobicity pocket of a neighbouring clamp molecule, by strong interaction between the ssDNA template of the primed DNA with two tyrosines (Tyr153 and Tyr154) in the pocket [93]. *Dr*β-clamp does not have these tyrosines, they are substituted by Ala151 and Val152, which may explain why the DNA failed to bind in the same manner in our protein. To be able to co-crystallise *Dr*β-clamp with DNA, oligonucleotides with different lengths and sequence should be tested.

DNA interacting residues

Co-crystallisation studies with β-clamp have shown that DNA is bound perpendicular to the plane of the protein-ring making the axis of each α-helix located in the inner ring of the sliding clamp perpendicular to the DNA as well [177]. This makes it easier for the clamp to slide along the DNA since it cannot interact directly with DNA minor or major grooves. The positions of positively charged residues on the β-clamp inner surface are only moderately conserved compared to *E. coli* β-clamp (38% identity and 43% similarity) (Figure 4, **manuscript II**), which indicates that the DNA backbone position is not vital. In the DNA complex structure of *Ec*β-clamp, Arg24 and Gln149, were identified as important residues for dsDNA interaction and necessary for clamp loading [93]. Arg24 is highly conserved in β-clamps and is identified in *Dr*β-clamp as Arg25. Gln149 is less conserved and is substituted by Glu147 in *Dr*β-clamp (Figure 2, **manuscript II**). The consequence of having a negatively charged residue in *Dr*β-clamp in the same position as Gln149 in *Ec*β-clamp is not known. A negative residue (Asp) is also found in *M. tuberculosis* [176] and *T. maritima* (unpublished) β-clamps at the same position making us believe it could also be involved in DNA interaction.

4.2.2 *Dr*ExoIII

D. radiodurans only possesses one gene encoding an AP-endonuclease (ExoIII) in its genome, while many other organisms possesses an additional AP-endonuclease, EndoIV, which is known to be inducible upon exposure to oxidative stress in bacteria [178-181]. Both ionising radiation and desiccation induces production of high amounts of ROS, thus it is peculiar that *D. radiodurans*, which is very resistant to both these genotoxic conditions does not have the EndoIV enzyme. Thus we were curious to know if the ExoIII enzyme possesses special properties, which enable efficient processing of AP-sites in *D. radiodurans* under stressful conditions.

Homology modelling indicates that *Dr*ExoIIIΔ22 also adopts a four-layered α/β-sandwich motif as seen in other members of the *Xth*-family of AP-endonucleases, and that all the catalytic residues known to participate in AP-site recognition are conserved.

We measured activity of the N-terminally truncated *Dr*ExoIIIΔ22 on substrate oligonucleotides with both AP-sites and 3´-unsaturated aldehydes and found that the enzyme processes both damages, and also exhibits 3´-5´-exonuclease activity, all on dsDNA.

Studies have shown that *D. radiodurans* has an optimal growth temperature between 25-35°C, but its natural habitat is not known. Here we show that *Dr*ExoIIIΔ22 has the ability to incise DNA over a very broad temperature and pH range. The enzyme was examined for optimal pH and temperature conditions, and interestingly it is highly active over the whole temperature and pH range tested (4 - 70°C and pH 5.5 – 10.5). We also found the enzyme to have a thermal stability around 45°C by DSC-scanning of the enzyme with $MgCl_2$ both present and lacking from the assay buffer. Taken together, we find it interesting that the enzyme has high activity at 4°C and high overall stability, thus possibly making this enzyme attractive for commercial biomolecular applications where high stability at ambient temperatures or high activity at low temperatures are needed.

3´ - 5´-exonuclease activity is affected by salt

Knowing that ExoIII from other species needs Mg^{2+} for AP-endonuclease activity and human and mouse APE1 have shown abrogated AP-endonuclease activity at salt concentration above 200 mM [182], we wanted to analyse how $MgCl_2$ and NaCl affected the AP-endonuclease activity of *Dr*ExoIIIΔ22. Our assays clearly proved that the enzyme needed $MgCl_2$ to perform AP-endonuclease activity since the activity was completely abolished when $MgCl_2$ was absent. This has been seen and described for exonucleases from other organisms [24, 62]. At high $MgCl_2$ concentration (5 mM) and salt conditions lower than 150 mM, both the AP-endonuclease and the 3´- 5´- exonuclease activity was present demonstrating that the enzyme continued to degrade the oligonucleotide beyond the AP-endonuclease cleavage site. The exonuclease activity post AP-endonuclease cleavage was only limited to 3-4 bases, since the exonuclease activity is only observed on dsDNA [63, 183] and can be initiated from both 3´ ends of the DNA duplex.

We also observed that the salt concentration strongly affects this 3´-5´-exonuclease activity. The exonuclease activity is decreased with increasing salt concentration, and was absent when the NaCl concentration exceeded 150 mM in presence of 5 mM $MgCl_2$. This has also been observed for *Ec*ExoIII, where increasing the ionic strength of the reaction lead to a decreased rate of exonuclease activity [184, 185]. Based on these analyses we concluded that *Dr*ExoIIIΔ22 possesses both AP-endonuclease and 3´- 5´-exonuclease activity and that the AP-endonuclease activity is highly dependent on Mg^{2+} as described for other exonucleases [24, 62], and that the exonuclease activity is very salt sensitive, thus the balance between these two enzymatic activities can be modulated by the experimental conditions.

Physiological salt concentrations in a *D. radiodurans* cell is believed to be around 150mM NaCl, suggesting that under normal circumstances the salt sensitivity for 3´-5´-exonuclease activity is not an issue. But during desiccation the cell will be exposed to increased intracellular salt concentrations and under these conditions the AP-site recognition and processing might be of more importance than the 3'-5' exonuclease activity in order to increase genome maintenance efficiency. By visualising the electrostatic surface potential of the homology model of *Dr*ExoIIIΔ22-apo and the Apo-form of APE1 (PDB: 4QHD) we found the active site of *Dr*ExoIIIΔ22 to be slightly less positively charged than in APE1. We believe it is possible that an increase in salt concentration will reduce the binding of the DNA 3´-end and instead the enzyme may favor interactions with the abasic site. The Mg^{2+} dependence of the AP-endonuclease activity and the salt sensitivity observed for the 3'-5' exonuclease activity also makes it interesting for commercial exploitation as it makes it possible to manipulate the enzyme activity by changing the buffer conditions for the reactions.

We found the enzyme to be in possession of two Trp residues (Trp231 and Trp245), which have been found to be indispensable for AP-endonuclease activity and the binding ability to the AP-site in other bacterial ExoIII [186]. Interestingly, both *Ec*ExoIII and APE1 only possess one of this Trp (Trp212 and Trp280, respectively). The presence of two Trp residues in *Dr*ExoIII might increase the efficiency of AP-site recognition, which would be an advantage since *D. radiodurans* is shown to have only one AP-endonuclease assuming that a highly efficient AP-site recognition is extremely important for the

organism in order to process lethal AP-sites during exposure to high doses or ionising irradiation or desiccation. However, this needs to be investigated further by mutational and kinetic studies.

5. Concluding remarks and future prospects

Here we have studied three different proteins from two different extremophiles, *Aliivibrio salmonicida* and *Deinococcus radiodurans*, all involved in different parts of DNA metabolism.

As discussed above when *A. salmonicida* attacks its host the production of ROS is part of the host organism's first line of defense. We found MutT from *A. salmonicida* capable of hydrolysing the oxidative damaged nucleotide, 8-oxodGTP, in the nucleotide pool at low temperatures, thus avoiding incorporation of oxidised nucleotides into genomic DNA, and assisting the bacterium in its fight against oxygen radicals which are produced by its host upon infection.

The studies on β-clamp and ExoIII from *D. radiodurans* indicates that they both have developed properties to support efficient replication (β-clamp) and DNA-repair (ExoIII) upon exposure to ionic radiation and desiccation. β-clamp by having an inner side of the ring with a more spread charge, which we believe can cause a less tight binding and thus a more efficient sliding on DNA compared to other structurally determined bacterial β-clamps. However, we still need information about the replication speed for *Dr*β-clamp and to perform mutational studies in order to confirm our hypothesis. ExoIII seems to favour AP-endonuclease activity over 3´-5´-exonuclease activity under conditions with high intracellular salt concentration in presence of magnesium as *e.g.* during desiccation. But also here additional studies are needed to confirm our hypothesis. Besides reaction rate analyses, we are also in need of high-resolution protein crystal structures to fully confirm and elucidate protein function, catalytic potential, movements and regulatory mechanisms.

In general we can conclude that our studies have shown that these three enzymes have optimised their molecular functions in order to maintain their respective organisms genome stability under extreme conditions like low temperature and exposure to high doses of radiation and desiccation. However in the case of ExoIII and β-clamp more studies are needed in order to pinpoint the molecular determinants, which are underlying their contributions for efficient genome maintenance in *D. radiodurans.*

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