Protein Expression and Purification

Studies of multifunctional DNA polymerase I from the extremely radiation resistant Deinococcus radiodurans: recombinant expression, purification and characterization of the full-length protein and its large fragment

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Abstract: Deinococcus radiodurans is a bacterium with extreme resistance to desiccation and radiation. Although the origins of this extreme resistance have not been fully elucidated, an efficient DNA repair machinery that includes the enzyme DNA polymerase I, is potentially crucial as part of a protection mechanism. Here we have cloned and performed small, medium, and large-scale expression of full-length D. radiodurans DNA polymerase I (DrPolI) as well as the large/Klenow fragment (DrKlenow). We then carried out functional characterization of 5’exonuclease and DNA polymerase activities of these proteins using gel-based and molecular beacon-based biochemical assays. With the same expression and purification strategy, we got higher yield in the production of DrKlenow than of the full-length protein, approximately 2.5mg per liter of culture. Moreover, we detected a prominent 5’exonuclease activity of DrPolI in vitro. This activity and DNA polymerase activity of DrKlenow are preferentially stimulated by Mg2+ at pH 8.0-8.5 and are reduced by addition of NaCl. Interestingly, both protein variants are more thermostable at pH 6.0-6.5. The characterization of DrPolI’s multiple functions provides new insights into the enzyme’s role in DNA repair pathways, and how the modulation of these functions is potentially used by D. radiodurans as a survival strategy.

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Dear Editor

Lisbon, March 8th, 2021

Please find enclosed the manuscript entitled “Studies of multifunctional DNA polymerase I from the extremely radiation resistant Deinococcus radiodurans: recombinant expression, purification and characterization of the full-length protein and its large fragment”, by Andreia Fernandes, Yvonne Piotrowski, Adele Williamson, Kelly Frade and Elin Moe, that we are pleased to submit for publication in Journal of Protein Expression and Purification.

Deinococcus radiodurans exhibits an outstanding resistance to ionising radiation and desiccation, and tolerates in the order of 200 times higher doses of radiation than other bacteria without loss of viability. The mechanisms underlying these properties are not known, but it is generally accepted that the DNA repair machinery plays an important part of it. In this context, we have been studying the Base Excision Repair (BER) pathway for several years, and here we present a functional characterisation of full length DNA polymerase I (DrPoll) and its Large fragment (DrKlenow), which is part of this pathway in D. radiodurans.

DNA polymerases play a central role in replication and genome maintenance processes. Poll consists of three independent domains: an N-terminal domain with predicted 5’ exonuclease activity, a 3’ exonuclease proofreading domain and a C-terminal domain with DNA polymerization activity. Our sequence analysis demonstrate that DrPoll comprises two fragments equivalent to the proteolysis products of the Escherichia coli DNA polymerase I: the small fragment (33KDa) which corresponds to the N-terminal domain (5´exonuclease domain), and the 69KDa Klenow fragment or large fragment (DrKlenow) which retains the 3’ exonuclease and polymerase domain. DrKlenow have been cloned and analyzed by other authors (Heinz et al., 2007). They demonstrated that DrKlenow is a DNA-dependent polymerase which possesses strand-displacement and lesion-bypass activities, all dependent on divalent metal ions.

Here, we present an expression optimization and purification of the recombinant full-length polymerase DrPoll. In addition, we have performed stability and functional analysis of DrPoll and DrKlenow to characterize the modulation of 5´exonuclease and DNA polymerase activities, to further understand the contribution of DrPoll in D. radiodurans radiation resistance.
We believe this work represents a significant contribution to the field of protein expression and purification as well as DNA repair and hope that the presented findings meet the high publication standards of your journal.

Thank you for considering this manuscript,

Yours sincerely

[Signature]

Elin Moe
Studies of multifunctional DNA polymerase I from the extremely radiation resistant Deinococcus radiodurans: recombinant expression, purification and characterization of the full-length protein and its large fragment

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Abbreviations: 6-Histag, hexa histidine-tag; aa, amino acids; BSA, bovine serum albumin; Ca2+, calcium; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; ssDNA, single strand DNA; dsDNA, double strand DNA; dNTPs, mix of deoxynucleotide triphosphates; HCl, hydrochloric acid; Mg2+, magnesium; Mn2+, manganese; NaCl, sodium chloride; nt, nucleotides; Ni2+, nickel; PAGE, polyacrylamide gel electrophoresis; TBE, Tris-Borate-EDTA buffer; OD
600, optical density at 600nm;

Highlights
- Deinococcus radiodurans DNA polymerase I (DrPoll) has optimum 5’ exonuclease activity at pH 8.5 and in the absence of NaCl
- DNA polymerase activity is optimal at pH of 8.5 and in the absence of NaCl
- DrPoll full-length and truncated Klenow fragment (DrKlenow) are more thermostable at pH 6.5-6.0

Abstract

Deinococcus radiodurans is a bacterium with extreme resistance to desiccation and radiation. Although the origins of this extreme resistance have not been fully elucidated, an efficient DNA repair machinery that includes the enzyme DNA polymerase I, is potentially crucial as part of a protection mechanism.

Here we have cloned and performed small, medium, and large-scale expression of full-length D. radiodurans DNA polymerase I (DrPoll) as well as the large/Klenow fragment (DrKlenow). We then carried out functional characterization of 5’exonuclease and DNA polymerase activities of these proteins using gel-based and molecular beacon-based biochemical assays. With the same expression and purification strategy, we got higher yield in the production of DrKlenow than of the full-length protein, approximately 2.5mg per liter of culture. Moreover, we detected a prominent 5’exonuclease activity of DrPoll in vitro. This activity and DNA polymerase activity of DrKlenow are preferentially stimulated by Mg2+ at pH 8.0-8.5 and are reduced by addition of NaCl. Interestingly, both protein variants are more thermostable at pH 6.0-6.5.

The characterization of DrPoll’s multiple functions provides new insights into the enzyme’s role in DNA repair pathways, and how the modulation of these functions is potentially used by D. radiodurans as a survival strategy.

Keywords: Deinococcus radiodurans, DNA polymerase I, DNA repair, 5’ exonuclease, recombinant protein production, thermostability.
Introduction

Deinococcus radiodurans is extremely resistant against ionizing and UV radiation, desiccation and oxidizing agents, mostly due to a highly resistant proteome and an efficient DNA repair system. The ability to fully reassemble a fragmented genome, through a massive DNA synthesis after exposure to high doses of radiation, makes this bacterium an interesting model to study DNA repair and proteins involved in DNA metabolism.

DNA polymerases play a central role in the replication and maintenance of the genome. In the D. radiodurans genome, the genes encode a DNA polymerase I (DrPolI), DNA polymerase III (DrPolIII) and DNA polymerase X (DrPolX). D. radiodurans deletion mutants of each DNA polymerase are radiosensitive, particularly DrPolI and DrPolIII mutants. A combined knockout of both DrPol and DrPolIII genes led to irrecoverable DNA synthesis and ultimately cell death after irradiation with 7000 Gray. DrPolIII has a potential key role in initiating DNA repair synthesis and DrPol in the subsequent steps, according to. Additionally, proposed a molecular mechanism, dependent on DrPol, for fast shattered chromosome reassembly in D. radiodurans, the extended synthesis-dependent strand annealing (ESDSA). These findings show that DrPol is essential for the extreme radiation resistance of D. radiodurans. But its role in DNA repair is still only beginning to be understood, further characterization of this multifunctional enzyme is needed.

DrPol consists of three independent domains: the N-terminal domain with predicted 5’ exonuclease activity, the 3’ exonuclease proofreading domain and a C-terminal domain with DNA polymerization activity. As per DNA polymerase convention, DrPol comprises two fragments equivalent to the proteolysis products of the Escherichia coli DNA polymerase I: the small fragment (33KDa) which corresponds to the N-terminal domain (5’ exonuclease domain), and the 69KDa Klenow fragment or large fragment (DrKlenow) that retains the 3’ exonuclease and the polymerase domain. DrKlenow was first cloned and analyzed by. They demonstrated that this is a DNA-dependent polymerase that also possesses strand-displacement and lesion-bypass activities, all dependent on divalent metal ions.

Here, we present the expression optimization and purification of the recombinant full-length polymerase DrPol. In addition, we have performed stability and functional analysis of DrPol and DrKlenow to characterize the modulation of 5’ exonuclease and DNA polymerase activities, to further understand the contribution of DrPol in D. radiodurans radiation resistance.

Materials and Methods

1. Molecular Cloning

The gene encoding full-length DrPol was amplified from D. radiodurans R1 strain genomic DNA (GeneBank ID: AE00513, AE001825). The nucleotides 1-105 from the annotated DR_1707 sequence (NCBI ID: 1800388) were excluded as bioinformatic analysis of the gene and protein sequence showed an annotation error. The longer version of the gene encoded a protein of 956 aa that was not successfully expressed. The corrected gene encodes the 921 aa protein and this correct annotation has been re-written on NCBI (NCBI ID: WP_034351289.1), with a molecular mass of 101.9KDa. The PCR reactions of 50ul contained 1x CG buffer, 400μM of dNTPs, 200nM of each primer, 3% dimethyl sulfoxide and 2units of Phusion High-Fidelity DNA Polymerase (ThermoFisher). The gene was amplified during two PCR reactions (first reaction primers:
PCR reactions were performed with an initial denaturation at 98°C for 5 min, 30 cycles of denaturation (98°C, 30 sec), annealing (66°C, 1 min) and extension (72°C, 2 min), followed by a final extension at 72°C for 7 min. The gene was inserted into the pDest14 expression vector according to GATEWAY cloning system (GE Healthcare) guidelines. The DrKlenow was subcloned (first PCR primers: FPKlef, RPDrPoll; second PCR primers: FDRalle; RPDrPoll; Table 1) and inserted into pDest14 vector. The DrKlenow sequence encodes amino acids 302-921 based on the prediction by\textsuperscript{5}, and corresponds to a molecular mass of 68.9KDa. Both constructs encode an N-terminal 6-Histag sequence followed by a TEV cleavage site that were included in the cloned genes via primer FPDrPoll, for DrPoll, and primer FPKlef, for DrKlenow (Table 1).

2. Small, Medium and Large-scale Expression

For DrPoll expression, a small-scale test expression was performed by transforming the constructs into three different \textit{Escherichia coli} strains: BL21(DE3)*, BL21(DE3) pLysS and BL21(DE3)* pRARE2. Colonies of each strain were inoculated in 5ml LB medium with ampicillin (200mg.ml\textsuperscript{-1}) and in addition with chloramphenicol (34mg.ml\textsuperscript{-1}) for BL21(DE3) pLysS and BL21(DE3)* pRARE2 cells. Expression was induced by adding 0.5mM isopropyl β-d-1-thiogalactopyranoside (IPTG) at an OD\textsubscript{600} of 0.6, and performed at 37°C for 3h. Harvested cells were dissolved in 200µl extraction buffer (50mM Tris–HCl pH 7.5, 150mM NaCl, 10mM imidazole, 5mM MgCl\textsubscript{2}, 0.1mg.ml\textsuperscript{-1} lysozyme, 1ug.ml\textsuperscript{-1} DNase) and lysed by five freeze-thaw cycles in liquid nitrogen at 22°C. After a centrifugation (15000xg, 15min, 4°C) the lysate (cytoplasmic fraction) was collected and the pellet (insoluble fraction) was resuspended with 50µl of 1% sodium dodecyl sulfate (SDS), then heated at 95°C for 5min. The obtained fractions were analyzed on Tris-glycine-SDS-PAGE gel (10% for DrPoll, 7% for DrKlenow), which was stained with Bio-Safe Coomassie (Biorad). Then, a 50mL temperature optimization expression experiment was performed with BL21(DE3) pLysS cells. In this experiment, the expression induction was performed with the addition of 0.5mM IPTG when the cultures reached an OD\textsubscript{600} of 0.6 as described above. The culture for the expression test at 18°C grew overnight, at 25°C for 6h and at 37°C for 3h, reaching a final OD\textsubscript{600} of 2-3. Cells were harvested and lysed as described above. The cytoplasmic fraction (3ml) and insoluble fraction were separated by centrifugation (13000xg, 40min, 4°C) and treated as described above. After identifying the optimum conditions for the expression of recombinant DrPoll, expression of both full-length and DrKlenow was performed with BL21(DE3) pLysS cells induced with 0.5mM IPTG for 3h at 37°C.

3. Purification of DrPoll and DrKlenow

After large-scale expression (4L of full-length protein and 1L of DrKlenow), cells were harvested by centrifugation (8670xg, 10min, 4°C) and resuspended using the extraction buffer (20ml buffer per liter of culture) with protease inhibitor cocktail III (Merck Millipore) and lysed as described above. The lysate was centrifuged (27216xg, 40 min, 4°C) and the recovered supernatant was loaded onto a HisTrap HP\textsuperscript{™} column (GE Healthcare) using an AKTApurifier (GE Healthcare) column which was pre-equilibrated with buffer A (50mM Tris-HCl pH 7.5, 150mM NaCl, 10mM imidazole). The target proteins were eluted with a gradient of 10-300mM imidazole gradient in buffer A and analyzed by Tris-glycine-SDS-PAGE. Target-containing fractions were pooled, dialyzed into 50mM Tris-HCl pH 7.5 and 150mM NaCl , and incubated with 1:20 TEV protease\textsuperscript{9} overnight at 4°C. After cleavage the sample was re-purified using a HisTrap HP\textsuperscript{™} column to
remove the cleaved tag. Fractions containing the protein without 6-Histag were loaded onto a HiTrap Heparin HP™ column which was equilibrated with buffer B (20mM Bis-Tris propane pH 6.5, 150 mM NaCl) and the proteins were eluted with a gradient of 150-1000 mM NaCl in buffer B. For DrPoll an additional size-exclusion chromatography in buffer B was performed using a Superdex 200 10/300RP column (GE Healthcare). The purity of the recombinant proteins was assessed by Tris-glycine-SDS–PAGE (7% for DrPoll, 10% for DrKlenow). Both proteins were concentrated using Amicon Ultra centrifugal filters (MerckMillipore) and flash frozen in liquid nitrogen for storage at - 80°C in buffer B.

4. Thermofluor Assay

The SYPRO orange (Invitrogen) based thermal shift assay was performed by using 2.5µg of protein, 10X of dye and 18µl of buffer solution (for buffer screen description, Supplementary S1.) in a 20µl mix. Each condition was subjected to 1°C increase per minute in a range of temperature from 25°C to 90°C. Fluorescence measurement was optimized as described by and performed in iQ5 Real Time PCR Detection System (Bio-Rad), equipped with a charge-coupled device (CCD) camera, by exciting at 490nm and recording emission at 575nm. The peak minimum of first derivative [d(RFU)/dT] of Relative Fluorescence Units (RFU) over temperature (T) was determined as melting temperature (Tm).

5. Activity gel-based endpoint assay

Substrates were prepared by annealing the oligonucleotides in equimolar amounts in 10mM Tris-HCl pH 8.5, heating at 95°C for 5min and then gradually cooling overnight. For the DNA polymerase activity assays, reactions consisted of 50nM prime-template dsDNA substrate (oligos P1, P2, Table 2), 50uM dNTPs, 5mM dithiothreitol, 200ug.ml⁻¹ BSA, 2% glycerol, 5mM metal ion (Mn²⁺, Mg²⁺ or Ca²⁺), NaCl (variable concentrations) and 20mM Bis-Tris propane (variable pH). For 5’nucleoside activity a single nicked dsDNA substrate was used consisting of oligo P1, S2 and S3 (Table 2). All 10µl reactions were initiated by adding 100ng of protein and incubated for 15min at 30°C. The reactions were stopped by adding 5µl quencher buffer (7M urea, 0.025% SDS, 0.025% bromophenol blue) and heating for 5 min at 95°C. The samples were analyzed by TBE-urea-PAGE (7M urea, 12% acrylamide). The gel was scanned for FAM with the FLA-5100 (Fujifilm) imager. Where relevant, the positive control E. coli Klenow fragment, was purchased from New England Biolabs.

6. Time-resolved molecular beacon assay

The time-resolved molecular beacon assay was used to measure polymerase activity. The substrate oligos (Molecular Beacon, Primer, Table 3) were prepared in equimolar amounts in 10mM Tris-HCl pH 8.5 and preincubated at 30°C for 30 min before the reaction. The reactions (50ul) consisted of 200nM molecular beacon substrate (modified from, Fig. 6 c), 0.2mM dNTPs, 5mM dithiothreitol, 200ug.ml⁻¹ BSA, 2% glycerol, 5mM Mg²⁺, NaCl (variable concentrations) and 20mM Bis-Tris propane (variable pH). A prior incubation at 30°C was performed and the reactions were then initiated by adding 250ng of enzyme. FAM fluorescence was measured by exciting at 485nm and recording emission at 520nm. The measurements were carried out on the microplate reader FLUOstar (Optima) at 30°C for 50min by using black no binding surface 96-
well plates (Corning). Through the plot of fluorescence as function of time, the slope of the fitted line to the initial linear region of the plot was determined and represented as polymerase activity.

Results

1. DrPol low expression and DrKlenow overexpression

For the full-length protein DrPol, we started with a small-scale test expression to screen three hosts: *E. coli* BL21(DE3)*, BL21(DE3) pLysS and BL21(DE3)* pRARE2. Although DrPol was not overexpressed, we observed a ~100KDa protein band in the insoluble fraction of BL21(DE3) pLysS and BL21(DE3)* pRARE2 cells (Fig. 1 a). Thus, these strains were selected for a medium-scale test expression at different temperatures: 18, 25 and 37°C. In this experiment a ~100KDa band, corresponding to DrPol, was identified in the cytoplasmic and insoluble fraction from BL21(DE3) pLysS cells, at 37°C (Fig. 1 b). This was not observed in the non-induced cells. For DrKlenow, a medium-scale expression experiment with BL21 pLysS cells was also performed, and the protein was overexpressed in both soluble and insoluble form at 37°C (Fig 1 c). Therefore, for large-scale expression of the two proteins, we used BL21(DE3) pLysS cells induced with 0.5mM IPTG, incubated at 37°C for 3h and we obtained DrPoll and DrKlenow in soluble form.

2. Purification of DrPol and DrKlenow

DrPol and DrKlenow were expressed with a 6-Histag and TEV cleavage site in the N-terminus. Thus, both proteins were subjected to an initial three-step purification procedure: immobilized metal ion affinity chromatography (IMAC) using a HisTrap column, TEV protease cleavage followed by a second IMAC. In the first IMAC, DrPol was eluted at ~120mM imidazole (Fig. 2 a). The protein’s low affinity to the column decreased the purification efficacy. DrKlenow was eluted at ~250 mM imidazole (Fig. 2 b). To remove DNA from the samples, a HiTrap heparin chromatography step was included, and we obtained > 95% pure DrKlenow (eluted at 700mM NaCl, Fig. 2 d) while the DrPol sample (400mM NaCl) still contained contaminants. Therefore, for DrPol purification an additional gel filtration step was included (Fig. 2 c) and we confirmed that it is a monomeric protein. DrPoll production was affected by the low gene expression and low affinity of the protein to the HisTrap column, so the yield was only 0.5mg per liter of culture. Regarding DrKlenow, we used a similar expression and purification approach and we got 5 times higher production yield, 2.5mg per liter of culture.

3. DrPoll and DrKlenow are thermostable at pH 6.0-6.5

To assess the thermal stability of the proteins we performed Thermofluor assay, by screening both buffer pH and salt concentration. Regarding DrPoll, curves of SYPRO orange fluorescence as a function of temperature presented the expected thermal denaturation profile in a pH range from 6.0 to 8.5 at NaCl concentrations ≥ 150mM (Supplementary S2). Based on the observed Tm and curve shapes, the most stabilizing buffers considered are potassium phosphate pH 6.0, sodium phosphate pH 6.5, sodium cacodylate pH 6.5 and Bis-Tris propane pH 6.5 (Table 4). DrKlenow was stable over a broader pH range, from pH 5.0 to 9.5 (Supplementary S3). The best
buffers observed were sodium citrate pH 5.5, potassium phosphate pH 6.0, sodium phosphate pH 6.5 and sodium cacodylate pH 6.5 (Table 4). Thus both DrPolI and DrKlenow are stabilized in phosphate buffers at pH 6.0 and 6.5, in sodium cacodylate and Bis-Tris propane at pH 6.5. Both proteins were eventually purified and stored in 20 mM Bis-Tris propane pH 6.5 with 150 mM NaCl (Fig. 3) as this buffer also provides a wide buffering range for activity assays while avoiding future issues with protein crystallization trials (phosphate buffers) or toxicity (cacodylate).

4. **Strong 5´exonulease activity of DrPolI**

To assess DNA polymerase activity, a primer extension assay was performed at pH 8.5 using a linear dsDNA substrate that consists of a 15nt labelled primer and 40nt template (Fig. 4 a). It was observed that the DNA substrate was degraded by the full-length protein (Fig. 4 b). Thus, nuclease activity of DrPolI was indicated during the evaluation of its polymerase function *in vitro*. Sequence comparison with other bacterial DNA polymerases I indicate that DrPolI does not possess 3´exonuclease activity, the catalytic amino acids from nuclease segments identified by Bernad *et al.* (1989) and named Exo I, Exo II, Exo III, are not conserved (Supplementary S4). Therefore, we assessed whether it was the predicted 5´exonuclease activity that caused DNA degradation. The 5´exonuclease activity assay, with a single nicked dsDNA substrate (Fig. 4 a), showed that DrPolI is degrading the substrate at the 5´ nick, in the presence or absence of dNTPs (Fig. 4 c). This exonuclease activity was absent from the DrKlenow fragment (Supplementary S5), confirming that DrPolI full-length possesses 5´exonuclease activity, a function that is associated to its N-terminal domain. To avoid interfering nuclease activity, the DrKlenow enzyme was used in later experiments for evaluation of polymerase activity.

5. **5´exonuclease activity decreases at lower pH and increasing NaCl concentration**

To characterize the 5´exonuclease activity of the full-length protein, which is the prominent activity of DrPolI *in vitro*, we analyzed the effect of pH, NaCl concentration and metal ions. The reactions were set up (1) with buffers at pH 8.5, 8.0, 7.7, 6.9, 6.5, 6.0, 5.5; (2) with 0, 50, 150, 250 and 500mM of NaCl, and (3) 5mM of Mn$^{2+}$, Mg$^{2+}$ and Ca$^{2+}$. The results showed that DNA digestion by DrPolI decreases at pH < 8.0 (Fig. 5 a) and NaCl concentration > 50mM (Fig. 5 b). Moreover, this activity is dependent on Mg$^{2+}$ (Fig. 5 c).

6. **DNA polymerase activity decreases at lower pH and increasing NaCl concentration**

DrKlenow, that only retains the 3´ exonuclease and the DNA polymerase domain, was first analyzed by$^8$. They demonstrated that the DrKlenow catalyzes DNA elongation in the presence of dNTPs and preferentially in the presence of Mg$^{2+}$, and its DNA polymerase function is active until 50°C. To further characterize DrKlenow DNA polymerase activity, we analyzed the influence of pH and salt by using a molecular beacon assay (Fig. 6 c). The reactions were set up (1) with buffers at pH 8.5, 8.0, 7.7, 6.9, 6.5, 6.0 and (2) with 0, 50, 150, 250 and 500mM of NaCl. The molecular beacon assay results showed that DrKlenow is optimally active at pH 8.5 without salt (Fig. 6 a, 6 b). Furthermore, the DNA polymerase activity decreases considerably at pH < 8.0 (Fig. 6 a) and is completely absent at pH < 6.5. These results were also verified on gel (Supplementary S6) by using a primer-template DNA substrate confirming that the detected activity is indeed due to polymerase-related strand displacement and not residual nuclease activity (Fig. 4 a).
Discussion

We have cloned and recombinantly produced DrPolI and its 5’ truncated large fragment, DrKlenow. DrPolI and DrKlenow show different levels of expression in *E. coli* BL21(DE3) pLysS at 37°C. DrKlenow is overexpressed but the full-length protein’s substantially lower expression level implies that DrPolI size and extra domain negatively impact the expression process. DrKlenow was previously expressed and purified with an N-terminal 6-Histag by⁸. For DrPolI, through preliminary analysis of Swiss Model¹³ ¹⁴ ¹⁵ ¹⁶ predicted structure, based on the template 1taq.1.A (*Thermus aquaticus* DNA polymerase I - 48% identity and 56% similarity), we anticipated that the N-terminal part of the protein would be exposed to the surroundings (Supplementary S7). However, the low binding affinity of DrPolI, to the Ni³⁺ column in the first purification step, implies that the tag might be partially sequestered, leading to a weak interaction with the column resin. The protein’s C-terminus is also predicted to be exposed (Supplementary S7), hence a suitable alternative approach could be to insert the tag there for affinity chromatography purification purposes.

Regarding enzymatic activities, previous DrKlenow characterization studies⁸ demonstrated that this fragment catalyzes DNA synthesis because it possesses DNA polymerase and strand-displacement activities. DNA polymerization is active until 50°C, is dependent on dNTPs and is activated preferentially by Mg²⁺. Strand-displacement DNA synthesis is stimulated by Mg²⁺ and weakly affected by Mn²⁺. Also demonstrated that increasing Mn²⁺ levels decreases DrKlenow selectivity, while lesion bypass is activated by Mn²⁺. This supports the hypothesis of Mn²⁺ as a modulator of *D. radiodurans* enzymes. In our study we further showed that DrKlenow polymerase activity is mostly active at pH 8.5, is completely inhibited at pH < 6.5 and decreases with increasing NaCl concentration. DrKlenow is predicted to lack 3’ exonuclease activity as the conserved nuclease catalytic residues are absent from the 3’ exonuclease domain sequence, which is also seen in other family I DNA polymerases such as the *T. aquaticus* PolI which do not have 3’ exonuclease activity¹⁷.

Regarding the full-length protein, we showed that the 5’ exonuclease activity is optimal at pH 8.5-8.0 and is dependent on divalent metals, specifically Mg²⁺. This metal ion may be important in the DNA binding by direct interaction with the phosphodiester bond to be cleaved.¹⁸ Moreover, the strong 5’ exonuclease activity of DrPolI *in vitro* might indicate an important role *in vivo*. Gutman *et al.* (1994) previously demonstrated that the DNA repair ability was completely restored in *D. radiodurans* DrPolI deletion mutants expressing full-length *E. coli* DNA polymerase I (EcPolI). However while complementation with *E. coli* Klenow fragment improved resistance, this was not fully restored in strain 6R1A (*D. radiodurans* targeted insertion mutant). An indication that 5’ exonuclease domain is important in DNA damage response of *D. radiodurans*. DrPolI as a 5’ exonuclease might have a relevant role in processing damaged DNA fragments. Furthermore, 5’ exonuclease and DNA polymerase activities are better under neutral-alkaline pH and significantly decrease at NaCl concentration > 50mM. Interestingly, DrPolI and DrKlenow are more thermostable under neutral-alkaline pH and higher ionic strength, an indication of an ambivalent relation between enzymes activity and stability.

Overall, DrPolI possesses DNA polymerase, DNA strand displacement, 5’ exonuclease and lesion bypass activities. Upon damage of DNA, DNA polymerase, strand displacement and lesion bypass activities are critical functions in the DNA repair balance, with the 5’ exonuclease activity in DNA degradation and repair counterbalance. Moreover, although strand displacement, DNA polymerase and 5’ exonuclease are mostly active in the presence of Mg²⁺, lesion bypass is
activated by Mn$^{2+}$. It has been observed that *D. radiodurans* accumulates high levels of intracellular Mn$^{2+}$, a feature which is associated with its extraordinary ability to survive high levels of ionizing irradiation. Mn$^{2+}$ positively affects other DNA polymerases in *D. radiodurans*, DrPolIII and DrPolX. The DrPolX DNA polymerase and 3’ exonuclease activities have strong Mn$^{2+}$ dependence and DrPolIII (DnaE) polymerase activity is also strongly activated by this metal ion. Moreover, DrPolIII is known to be significantly stimulated by increasing ionic strength (200mM NaCl or KCl). On the contrary, we have shown that DrPoll and DrKlenow activities decrease significantly with increasing ionic strength. Therefore, our observations suggest that, besides alterations in intracellular Mg$^{2+}$ and Mn$^{2+}$, salt concentration may regulate the activity of *D. radiodurans* DNA polymerases, towards activation or inactivation depending on the state of the cell, pathway and/or the step of the repair process.

**Conclusion**

We present here the first recombinant expression, purification and preliminary characterization of the full-length DNA polymerase I from the extremophile *D. radiodurans*. The results showed that the enzyme is not only a DNA polymerase but also a 5’ exonuclease. As a multifunctional protein DrPolI is probably involved in many processes, including DNA fragments assembly, DNA synthesis as well as processing of damaged DNA. The modulation of its functions is critical in DNA repair and is influenced by intracellular factors such as divalent metal ions and salt concentration in *D. radiodurans* cells.

**Acknowledgement**

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**References**


## Tables

### Table 1. Primer sequences for the cloning of DrKlenow and DrPoll

<table>
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<th>Name</th>
<th>Sequence 5’ -&gt; 3’</th>
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<tr>
<td>FPDrPolI</td>
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### Table 2. DNA substrates oligonucleotide sequences for gel activity assays of DrPoll, * FAM (6-Carboxyfluorescein).

<table>
<thead>
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<td>ATTGAGTGGAACAAAGTATCGTGATGAGTATTGGTGGATA</td>
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<tr>
<td>P2</td>
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<td>CGATACTTTGTCCACTCAAT *</td>
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### Table 3. Oligonucleotides sequences of molecular beacon and primer for the molecular beacon substrate to determine DNA polymerase activity. * FAM (6-Carboxyfluorescein), Δ Dabcyl

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<td>Molecular Beacon</td>
<td>GGCCCGT Δ ACGAGGAAAGGACATCTTCTAGCAT * ACGGCCCCGTCAATTCATGCCAGTGCAAGTCTGCAGAAATTTCACCGACCAC</td>
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<td>Primer</td>
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Table 4. Tm values: most stabilizing buffers.

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<td>Bis-Tris propane pH 6.5</td>
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**Figures**

**Figure 1. Expression of DrPoll (101.9KDa) and DrKlenow (68.9KDa):**

a. 10% SDS-PAGE of 5mL DrPoll test expression to screen *E. coli* strains, all samples were induced with 0.5mM IPTG, expression for 3h at 37°C. 
b. 7% SDS-PAGE of 50mL DrPoll test expression to screen different temperatures by using BL21(DE3) pLysS cells, expression for 3h in the experiment at 37°C, for 6h at 25°C, overnight at 18°C. 
c. 7% SDS-PAGE of 50mL DrKlenow expression by using BL21(DE3) pLysS cells, expression induced with 0.5mM IPTG for 3h at 37°C. 
s - cytoplasmic fraction, i - insoluble fraction, Control - non induced cells, arrow - indicates the expected size position of DrPoll band.

**Figure 2. Purification results of DrPoll (101.9KDa) and DrKlenow (68.9KDa):**

a. DrPoll: 7% SDS-PAGE analysis of first purification with HisTrap column. 
b. DrKlenow: 10% SDS-PAGE analysis of first purification with HisTrap column. 
c. DrPoll: after last purification step (gel filtration). 
d. DrKlenow: after last purification step (HiTrap heparin purification). 
F - flow-through, W - wash (15mM imidazole), E - elution (120/250mM imidazole), p - pure protein (10ug).
Figure 3. Thermal shift assay curves: DrPoll and DrKlenow in buffer 20mM Bis-Tris propane pH 6.5, 150 mM NaCl (final purification buffer).

Figure 4. Gel-based activity assays: a. schematic representation of the DNA substrates, top - DNA polymerase assay, bottom - 5’ exonuclease assay. b. and c. 7M/12% urea PAGE for the analysis of DrPoll functions, the activity assays were performed for 15min at 30°C and pH 8.5. b. DNA polymerase activity assay (EcKlenow - *E. coli* Klenow fragment, New England Biolabs, M0210). c. 5’ exonuclease activity assay. + present, - absent.
Figure 5. DrPoll 5’ exonuclease activity characterization, experiments performed with 100ng of protein and the single nicked dsDNA substrate represented in Fig 4 a. a. pH influence, experiment performed with 50mM NaCl and 5mM Mg^{2+}. b. NaCl influence, experiment performed at pH 8.0 and 5mM Mg^{2+}. c. metal ions dependence, experiment performed at pH 8.0, with 50mM NaCl and 5mM each metal. + present, - absent.

Figure 6. DrKlenow DNA polymerase activity characterization, experiments performed with 250ng of protein and in duplicate. The increase in fluorescence due to polymerase activity was measured as relative fluorescence units (RFU) over time, and the polymerase activity is represented as mRFU per minute (mRFU/min). a. pH influence, experiment performed with 5mM Mg^{2+}. b. NaCl influence, experiment at pH 8.5 and with 5mM Mg^{2+}. c. schematic representation of the molecular beacon substrate.
Supplementary information

Studies of multifunctional DNA polymerase I from the extremely radiation resistant *Deinococcus radiodurans*: recombinant expression, purification and characterization of the full-length protein and its large fragment

Fernandes, A.1,2, Piotrowski, Y.2, Williamson, A.2,3, Frade, K.1, and Moe, E.1,2

1 Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal
2 UiT - The Artic University of Norway, Tromsø, Norway
3 University of Waikato, Hamilton, New Zealand

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Supplementary Figure 1. Thermofluor assay buffer screen description (reference: 50mM Tris-HCl pH7.5, 150mM NaCl)
Supplementary Figure 2a. DrPolI thermofluor assay buffer screening (results from well A1-A12 and B1-B12)

Supplementary Figure 2b. DrPolI thermofluor assay buffer screening (results from well C1-C12 and D1-D12)
Supplementary Figure 2c. DrPolI thermofluor assay buffer screening (results from well E1-E12 and F1-F12)

Supplementary Figure 2d. DrPolI thermofluor assay buffer screening (results from well G1-G12 and H1-H12)
Supplementary Figure 3a. DrKlenow thermofluor assay buffer screening (results from well A1-A12 and B1-B12)

Supplementary figure 3b. DrKlenow thermofluor assay buffer screening (results from well C1-C12 and D1-D12)
Supplementary figure 3c. DrKlenow thermofluor assay buffer screening (results from well E1-E12 and F1-F12)

Supplementary figure 3d. DrKlenow thermofluor assay buffer screening (results from well G1-G12 and H1-H12)
Supplementary Figure 4. Bacterial DNA polymerase I sequences alignment of the 3’ exonuclease domain catalytic region. Alignment by ClustalW (European Bioinformatics Institute). Amino acids for 3’ exonuclease activity indicated by red rectangle. PolI – DNA polymerase I, Aa - A. aeolicus, Bb – B. burgdorferi, Dr - D. radiodurans, Ec - E. coli, Ta - T. aquaticus, Tf - T. thermophilus.
Supplementary figure 5. 5’ exonuclease activity assay with DrKlenow. Experiments performed with 100ng of protein and the single nicked dsDNA substrate represented in Fig 4 a, at pH 8.5 and 5mM Mg²⁺. + present, - absent.

Supplementary Figure 6. DrKlenow DNA polymerase activity dependence on pH. Experiments performed with 100ng of protein, the prime-template dsDNA substrate represented in Fig 4 a, and with 5mM Mg²⁺. + present, - absent.
Supplementary figure 7. Swiss Model DrPoll predicted structure (template 1taq.1.A). Swiss Model homology model of DrPoll structure based on *T. aquaticus* DNA polymerase I structure (1taq.1.A). N-terminal residues 11-14 in red. C-terminal residues 916-920 in blue.