Supplementary Information

The crystal structure of a natural DNA polymerase complexed with mirror DNA

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Methods

Materials. All DNA polymerases except Dpo4 were purchased from New England Biolabs (USA). D-DNA oligonucleotides were purchased from Bioneer (Korea). L-DNA oligonucleotides were synthesized on the 1 μmol scale by Mermaid-4 DNA/RNA synthesizer (Bioautomation, USA) using standard phosphoramidite chemistry. L-DNA phosphoramidites were purchased from ChemGenes (USA). Fluorescein phosphoramidite was purchased from Glen Research (USA). L-dTTP was purchased from TriLink BioTechnologies (USA). D-dNTPs were purchased from Promega (USA).

Expression and purification of Dpo4. The pET21b plasmid carrying codon optimized *S. solfataricus* Dpo4 was kindly gifted from Prof. Duhee Bang (Yonsei University, Korea). The recombinant plasmid was transformed to C41(DE3) expression host. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside at 18 ºC for 21 hours. Dpo4 was purified following the previously reported procedure [1] using HisTrap and HiTrap monoS columns. Purified proteins were concentrated to 11 mg/mL with 10K cut-off centrifugal filter in the buffer 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT. The sequence of pET21b-DPO4-His6 plasmid used for expression of Dpo4 in this study is shown below. The coding sequence of Dpo4-His_6 is shown in red

pET21b–DPO4–His_6

TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCG
TGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCAC
GTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTA
CGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAG
ACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAAC
AACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTT
AAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTTCAGG
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TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTC
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CGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCT
CAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAA
GTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATA
CACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGA
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AACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTT
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GCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAAT
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GAGCGCTGCCGGCACCTGTCCTACGAGTTGCATGATAAAGAAGACAGTCATAAGTGCGGCGACGAT
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TGTTCGTTTCTCTAAATTCATCGAAGCTATCGGTCTGGACAAATTCTTCGACACCCTCGAGCACCACC
ACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCG
CTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGG
AGGAACTATATCCGGAT

Gel mobility shift assay. Fluorescein-labeled primer (GCT ACG ACT CAC TAT GGA CG, 100 nM) and template (AAA AAA AAA ACG TCC ATA GTG AGT CGT AGC, 100 nM) in Thermopol buffer (20 mM Tris-HCl, 10 mM$(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100 pH 8.8@RT, New England Biolabs, USA) were heated to 95°C for 5 min and annealed to form fluorescein-labeled DNA duplexes by cooling to room temperature (25°C) for 10 min. To the solution of DNA duplex was added polymerases (terminal deoxyribonucleotide transferase (TdT) (100 unit), Deep Vent (exo-) DNA polymerase (28 unit), Dpo4 (10 μM), M-MuLV reverse transcriptase (2800 unit), Klenow fragment (3'→5' exo-) (70 unit), Taq DNA polymerase (70 unit), and Vent (exo-) DNA polymerase (28 unit)), and the mixtures were incubated for 30 min at room temperature. The mixtures were then analyzed by 6% non-denaturing PAGE run in 0.5×TBE (44.5 mM Tris-HCl, 44.5 mM Boric acid, 1 mM EDTA pH 8.0) at 100V. The bands were visualized using Chemidoc (Biorad, USA).

Fluorescence polarization assays. Fluorescein-labeled DNA duplexes (100 nM) composed of fluorescein-labeled primer (fluorescein-GGG GGA AGG ATT CC) and template (TCA CGG AAT

CCT TCC CCC) were mixed with the Dpo4 at varying concentrations (0–10 μM) in the Dpo4 reaction buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 5mM DTT, 10% glycerol, 0.1mM EDTA) and incubated at 4°C for 30 min. Binding affinity of Dpo4 for DNA duplexes were analyzed by fluorescence polarization values were measured using Appliskan (Thermo Fisher Scientific, USA). K_d values were estimated based on the non-linear regression to a one-site saturation ligand binding equation using SigmaPlot software (Systat, USA). For the competition experiment, To the solution of Dpo4 (1 μM) pre-mixed with the fluorescein-labeled L-DNA duplex (100nM) in the Dpo4 reaction buffer was added D-DNA duplex at varying concentrations (0 – 4 μM) and incubated at room temperature for 30 min. The fluorescence polarization values were measured using Appliskan (Thermo Fisher Scientific, USA). IC_{50} value was estimated based on the non-linear regression to a four parameter logistic curve equation using SigmaPlot software (Systat, USA). All data are the means of three independent experiments.

Primer extension. Prior to the extension assay, 5'-fluorescein-labeled DNA primers (fluorescein-GCT ACG AGC ACT ATG GA CG) and DNA templates (AAA AAA AAA ACG TCC ATA GTG AGT CGT AG C) were annealed by heating to 95°C for 2 min and slowly cooling to 4°C. The primer extension reaction was performed with 1 μM of primer/template in a buffer containing 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1 % TritonX-100, 1 mM dTTP and Dpo4 (1 μM). Reactions were carried out at 55 °C for 30 min, and terminated by adding an equal volume of 95 % formamide and 10 mM EDTA followed by incubation at 95°C for 5 min. Reaction products were resolved on 20 % denaturing PAGE in 7 M urea and visualized by iBright FL1000 (Invitrogen).

Isothermal titration calorimetry. Isothermal titration calorimetry of Dpo4 and DNA was measured on Nano ITC (TA Instruments, USA). Dpo4 and DNAs were dialyzed against ITC buffer, pH 7.5, containing 20mM HEPES, 100mM NaCl, 0.5mM EDTA, 0.5mM TCEP, and 10mM MgSO4. The optimized Dpo4 concentration was 25 μM and that of DNA was 125 μM. 5 μL DNA was automatically

titrated to Dpo4 solution in the sample cell and total 20 titrations were conducted with 300 sec intervals between each shot to reach the complete equilibrium. The total time of measurement was more than 6,000 sec for individual experiment. ITC of L-DNA to Dpo4 was carried out at 45°C and that of D-DNA to Dpo4 was at 15°C. The data was analyzed by the program Nanoanalyze (TA Instruments, USA) in which the binding ratios were fitted with the independent binding model.

Crystallization and structure determination. DPO4 and a DNA duplex formed with a primer (GGG GGA AGG ATT CC) and a template (TCA CGG AAT CCT TCC CCC) were incubated about 1:1.3 molar ratio at 4 °C. Crystals of Dpo4/D-DNA or Dpo4/L-DNA complexes was grown by using a sitting drop vapor diffusion method in drops containing 5 to 5 proportional volumes of protein/DNA solution and reservoir solution (0.1 M BIS-TRIS (pH 7.0), 0.1 M calcium acetate, 2% glycerol, and 9% w/v PEG3350) or reservoir solution (0.1 M Sodium formate, 0.05 M BICINE (pH 8.5), 10% w/v Polyethylene glycol monomethyl ether 5,000, and 1.1% benzamidine-HCl) at 19 °C, respectively. Crystals were quickly soaked in 0.1 M BIS-TRIS (pH 7.0), 20% PEG3350, 100mM CaOAC2, and 25% Glycerol for Dpo4/D-DNA crystals and 0.1 M Sodium formate, 0.05 M BICINE (pH 8.5), 10% w/v Polyethylene glycol monomethyl ether 5,000, 1.1% benzamidine-HCl and 25% glycerol for Dpo4/L-DNA crystals and were immediately flash-frozen in liquid nitrogen. Data were collected at the wavelength 1.0 Å or 0.9794 Å, respectively at the Pohang Accelerator Light Source beamline 5C (Pohang, Korea). The data set was reduced and scaled using HKL-2000 [2]. Dpo4/D-DNA and Dpo4/L-DNA crystals were crystalized in $P22_12_1$ and $P12_11$ and diffracted to a resolution of 2.60 and 2.36 Å, and solved at 2.60 and 2.36 Å, respectively. Phases for both complexes were determined through molecular replacement using the published DPO4 structure (PDB 2BQ3) [3] and initial model building was done using Autobuild within the PHENIX [4]. Model building and refinements were done using COOT [5] and PHENIX [4]. The final models were validated via MOLPROBITY [6]. The statistics are summarized in Table S2.

Accession numbers. The crystal structures have been deposited at the RCSB Protein Data Bank under the accession codes 6L84 for DPO4/D-DNA duplex and6L97for DPO4/L-DNA duplex.

Table S1. D- and L-DNA sequences tested for Dpo4 binding

Name	Sequence
GC-0	5′-AAAAAAAAAAAAAAAAAA-FAM*-3′
	3′-TTTTTTTTTTTTTTTTTT-5′
GC-33	5′-CAATGTCTAAACTGAAAG-FAM-3′
	3′-GTTACAGATTTGACTTTC-5′
GC-67	5′-GGACCGTGACTCCCTGAC-FAM-3′
	3′-CCTGGCACTGAGGGACTG-5′
GC-100	5′-GGGGGGGGGGGGGGGGGG-FAM-3′
	3′-CCCCCCCCCCCCCCCCCC-5′

*FAM denotes fluorescein.

Table S2. Data collection and refinement statistics

	Dpo4	
	D-DNA	L-DNA
Data Collection		
Space group	P 2 2_1 2_1	P 1 2_1 1
Unit cell (a, b, and c; Å)	52.8, 98.7, 100.8	85.9, 55.0, 96.6
(α, β, and γ; °)	90.0, 90.0, 90.0	90.0, 113.4, 90.0
Wavelength (Å)	1.0	0.9794
Resolution[a] (Å)	50–2.60 (2.64–2.60)	50–2.36 (2.44–2.36)
R_{merge}[a,b] (%)	12.2 (35.9)	12.5 (55.5)
Mean $I/\sigma(I)$[a]	20.6 (5.4)	28.5 (3.1)
Completeness[a] (%)	99.8 (99.8)	97.9 (95.3)
Redundancy[a]	11.3 (7.7)	4.6 (3.0)
Observed reflections (unique)	188,903 (16,695)	153,932 (33,537)
Wilson B-factor	35.1	50.5
Refinement		
R_{work}/R_{free}[c] (%)	18.2/22.6	23.0/26.6
Protein residues per asu	341	672
Water molecules per asu	108	115
Other ligands per asu	29	28
CA	3	-
Ramachandran plot		
Favored/allowed/outliers (%)	95.6/4.1/0.3	95.3/4..7/0.0
Rms deviations		
Bond lengths (Å)	0.003	0.006
Bond angles (°)	0.475	0.70
Average B-factor (Å^2)	42.7	66.8
Macromolecules	42.8	66.2
Ligands	52.8	74.4
Solvent	40.8	55.9
PDB code	6L84	6L97

[a] Numbers in parentheses indicate the highest resolution shell.

[b] $\mathbf{R}_{merge} = [\Sigma_{hkl} \Sigma_i |\mathbf{I} - \langle \mathbf{I} \rangle| / \Sigma_{hkl} \Sigma_I |\mathbf{I}| \times 100]$.

[c] $\mathbf{R}_{factor}/\mathbf{R}_{free} = \Sigma_{hkl} || \mathbf{F}_o | - | \mathbf{F}_c || / \Sigma_{hkl} | \mathbf{F}_o |$, where $\mathbf{F}_o$ and $\mathbf{F}_c$ are observed and calculated structure factors, respectively.

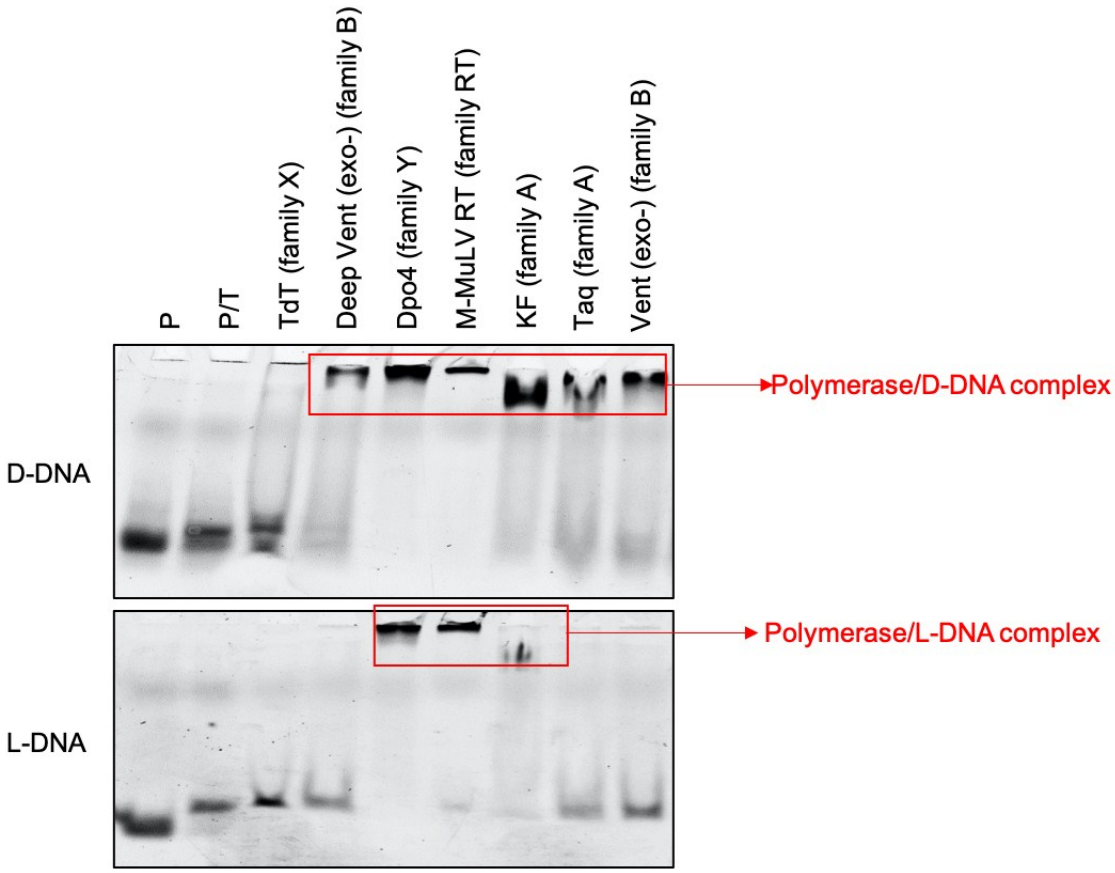

Figure S1. Native PAGE (6%) screening of polymerase binding to a D-DNA (top) or L-DNA (bottom) duplex. P and T denote primer and template, respectively.

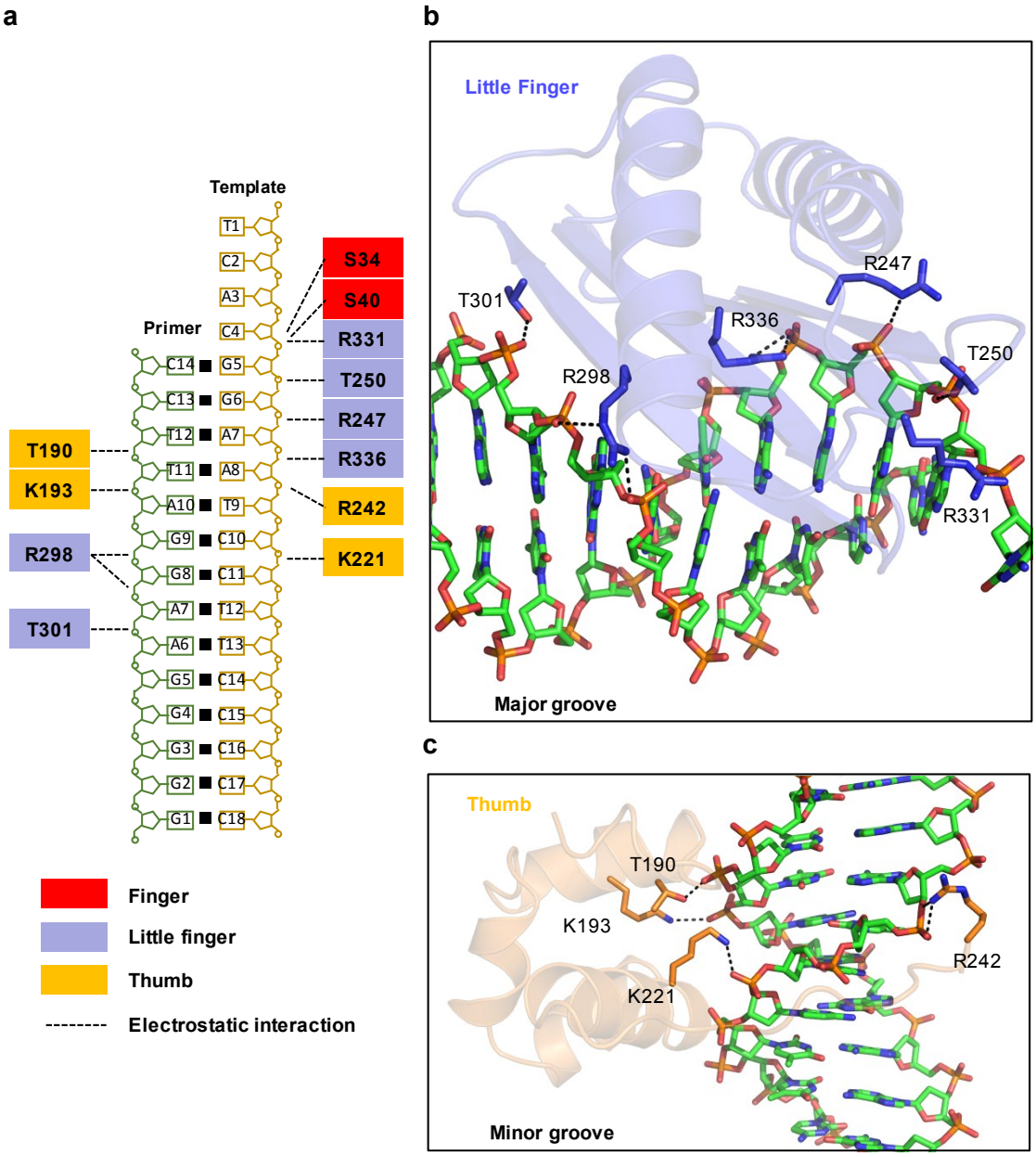

Figure S2. (a) Schematic diagram of the Dpo4–D-DNA interaction. (b) The structure of D-DNA interacting with the little finger domain of Dpo4. (c) The structure of D-DNA interacting with the thumb domain of Dpo4.

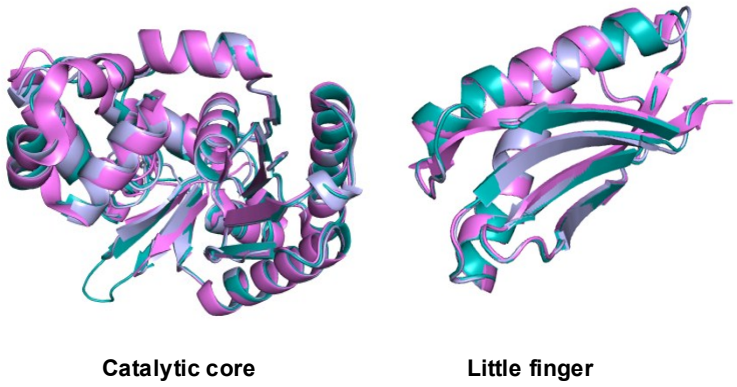

Figure S3. Structural alignment of catalytic core and little finger in the Dpo4/D-DNA duplex (turquoise), in the Dpo4/L-DNA duplex (pale blue), and in the apo-Dpo4 (pink) structures using PyMol [7].

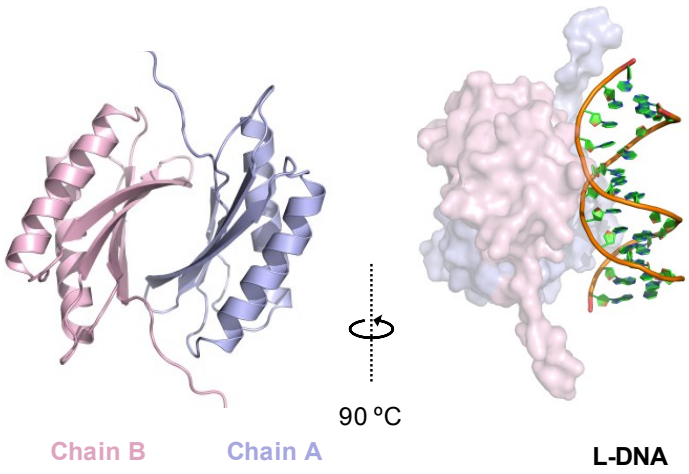

Figure S4. The structure of the dimerized little finger domains in the Dpo4/L-DNA complex, and its view rotated by 90°.

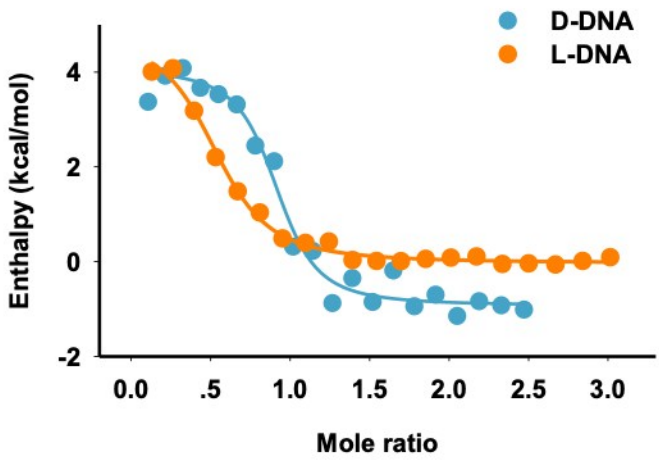

Figure S5. Binding stoichiometry curves estimated by isothermal titration calorimetry.

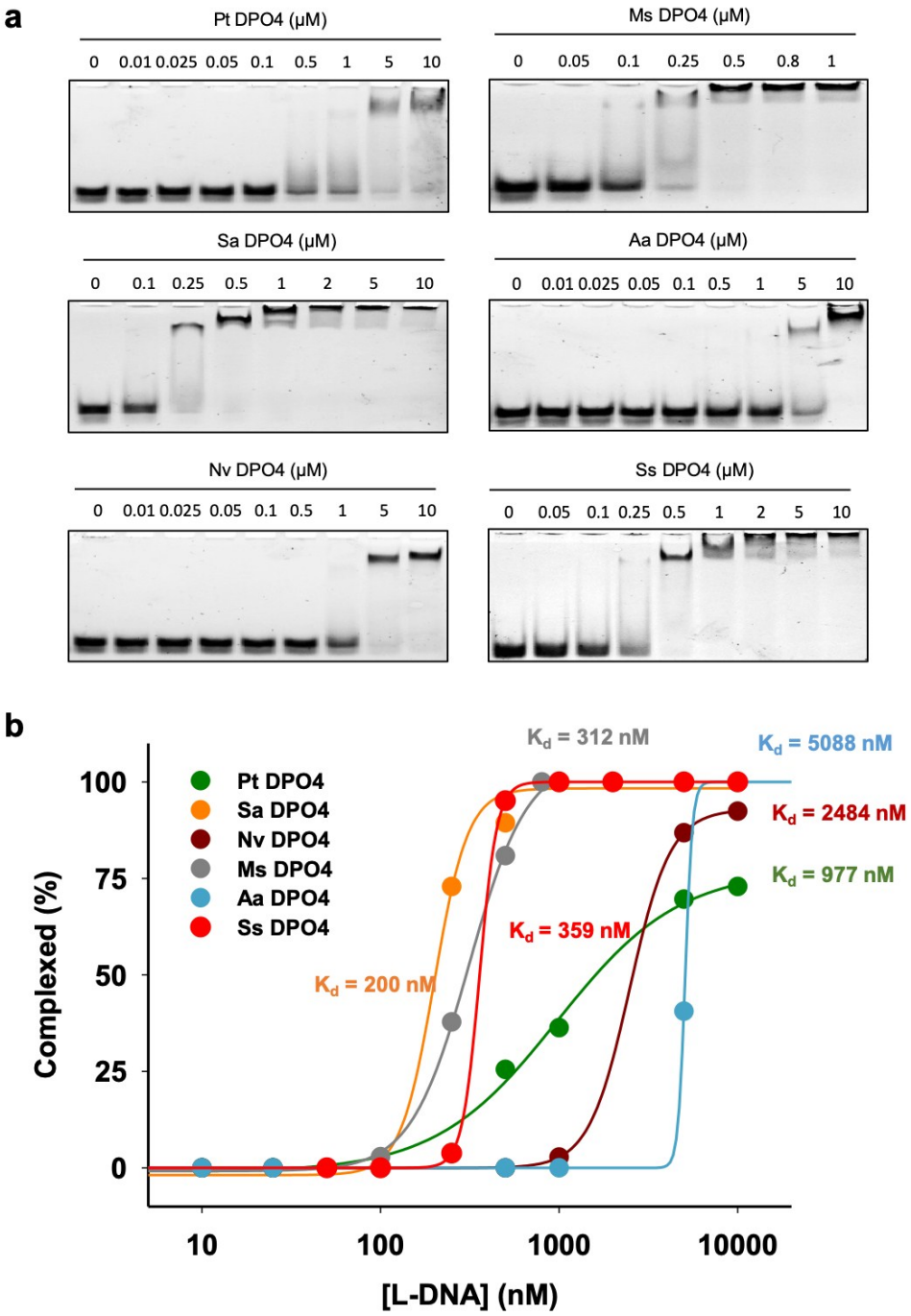

Figure S6. **(a)** 6% Native PAGE to estimate binding of L-DNA to Dpo4 from various archaea species at varying concentrations. **(b)** The K_d values were determined by quantification of the band intensity and non-linear regression to a four parameter logistic curve equation using SigmaPlot software (Systat, USA).

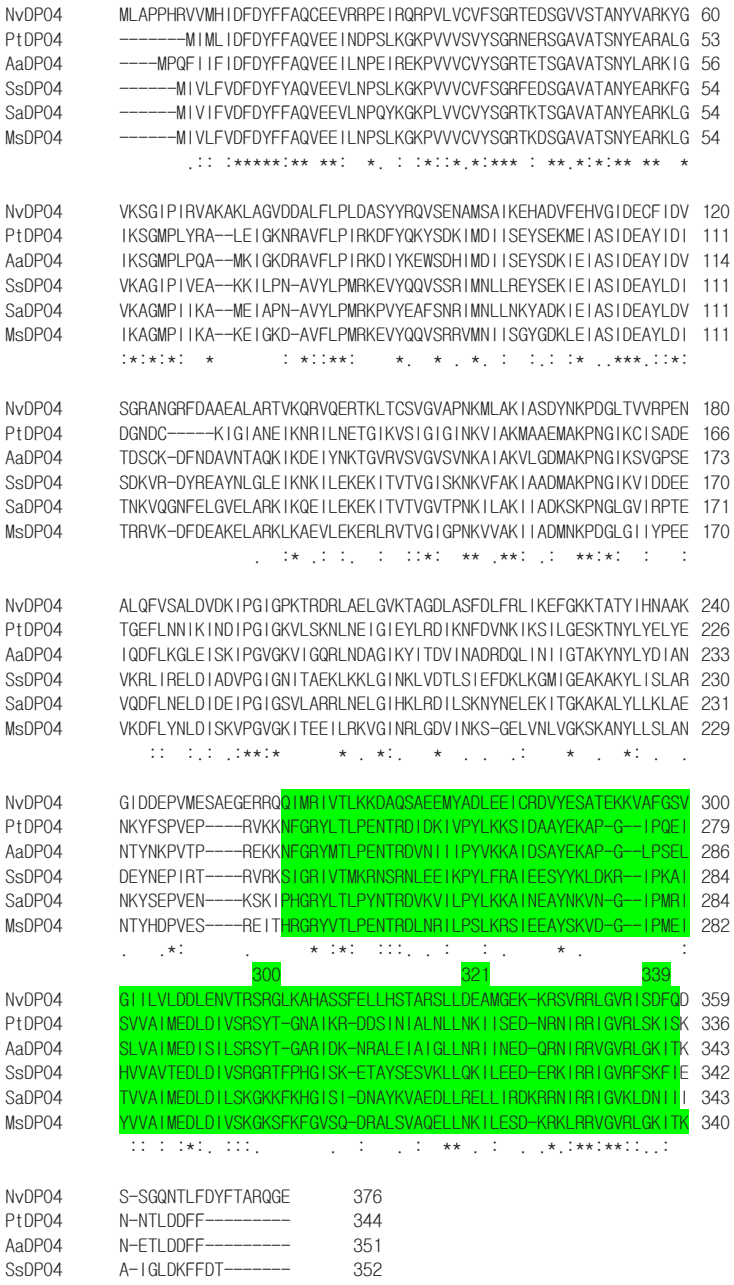

Figure S7. Sequence alignment of Dpo4 polymerases from six different archaeal species. The little finger domain was highlighted with green. The alignment was performed using the previously reported method [8].

References

[1] *Biochemistry*, **43**, 2106-2115 (2004)

[2] *Method Enzymol*, **276**, 307-326 (1997)

[3] *J Biol Chem*, **280**, 29750-29764 (2005).

[4] *Acta Crystallogr D*, **66**, 213-221 (2010).

[5] *Acta Crystallogr D*, **60**, 2126-2132 (2004)

[6] *Acta Crystallogr D*, **66**, 12-21 (2010).

[7] Schrodinger, LLC, The PyMOL Molecular Graphics System, Version 1.8, 2015.

[8] *Bioinformatics*, **23**, 2947-2948 (2007)