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## Characterization and application of a family B DNA polymerase from the hyperthermophilic and radioresistant euryarchaeon *Thermococcus gammatolerans*

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4 **Characterization and application of a Family B DNA polymerase from the**  
5  
6 **hyperthermophilic and radioresistant euryarchaeon *Thermococcus***  
7 ***gammatolerans***  
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62  
63 **Abstract**  
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65 *Thermococcus gammatolerans* is anaerobic euryarchaeon which grows optimally  
66 at 88°C and its genome encodes a Family B DNA polymerase (Tga PolB). Herein, we  
67 cloned the gene of Tga PolB, expressed and purified the gene product, and  
68 characterized the enzyme biochemically. The recombinant Tga PolB can efficiently  
69 synthesize DNA at high temperature, and retain 93% activity after heated at 95°C for  
70 1.0 hr, suggesting that the enzyme is thermostable. Furthermore, the optimal pH for  
71 the enzyme activity was measured to be 7.0-9.0. Tga PolB activity is dependent on a  
72 divalent cation, among which magnesium ion is optimal. NaCl at low concentration  
73 stimulates the enzyme activity but at high concentration inhibits enzyme activity.  
74 Interestingly, Tga PolB is able to efficiently bypass uracil in DNA, which is distinct  
75 from other archaeal Family B DNA pols. By contrast, Tga PolB is halted by an AP  
76 site in DNA, as observed in other archaeal Family B DNA polymerases. Furthermore,  
77 Tga PolB extends the mismatched ends with reduced efficiencies. The enzyme  
78 possesses 3'-5'exonuclease activity and this activity is inhibited by dNTPs. The DNA  
79 binding assays showed that Tga PolB can efficiently bind to ssDNA and primed DNA,  
80 and have a marked preference for primed DNA. Last, Tga PolB can be used in routine  
81 PCR.  
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105 **Keywords:** Archaea; DNA polymerase; DNA replication; Routine PCR  
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## Introduction

DNA polymerase (pol) is a ubiquitous enzyme that replicates DNA in all living cells. Since the first DNA pol was discovered and characterized from *Escherichia coli* in the 1950s by Arthur Kornberg, a large number of DNA pols have been biochemically and structurally characterized from diverse organisms. Currently, DNA pols have been grouped into six families: A, B, C, D, X, and Y, based on amino acid sequence similarities [1]. In addition, reverse transcriptase has been proposed as the seventh family since it can synthesize DNA [2]. Family B DNA pols have been found in bacteria, archaea, eukarya, and viruses/phages. A typical characteristic of most of Family B DNA pols is that they possess 3'-5' exonuclease activity in addition to polymerase activity [3; 4], which can remove a misincorporated nucleotide opposite a template strand, thus enhancing fidelity approximately 100-fold.

As the third life, Archaea are a simplified version of eukarya with respect to DNA metabolism [5; 6; 7]. Currently, DNA pols of Family B, D, and Y have been found in Archaea [8]. Family B DNA pols are ubiquitous in both *Euryarchaeota* and *Crenarchaeota*, which are two major branches of Archaea. In addition to possessing five conserved motifs, archaeal Family B DNA pols have a unique uracil binding motif [9; 10], which can recognize uracil and hypoxanthine bases derived from deamination in template DNA. Replication of uracil and hypoxanthine before repair leads to GC to AT mutation and AT to GC mutation, respectively. However, replication by archaeal Family B DNA pols would be stalled if deaminated base is encountered with these pols [11; 12]. Such an error avoidance mechanism would be expected to be evolutionary favorable for hyperthermophilic Archaea thriving in high-

178  
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180  
181 temperature environments where the rates of deamination are increased. Hence, the  
182  
183 recognition of these pro-mutagenic lesions and the following replication stop  
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185  
186 observed in archaeal Family B DNA pols might be a specific adaptation of  
187  
188 hyperthermophilic Archaea to their hostile environments.  
189

190  
191 As an important branch of euryarchaea, *Thermococcus* thrives in high-  
192  
193 temperature environments, including deep-sea hydrothermal vents and hot springs.  
194  
195 Increasing numbers of *Thermococcus* species have been isolated and identified [13].  
196  
197 Since the first archaeal Family B DNA pol was reported from *Thermococcus litoralis*  
198  
199 [14], a dozen of archaeal Family B DNA pols have been biochemically characterized  
200  
201 from *Thermococcus* species: *Thermococcus* 9°N-7 [15], *Thermococcus* *celer* [16],  
202  
203 *Thermococcus* *fumicolans* [17], *Thermococcus* sp. NA1 [18], *Thermococcus*  
204  
205 *peptonophilus* [19], *Thermococcus* *zilligii* [20], *Thermococcus* *aggregans* [21],  
206  
207 *Thermococcus* *celer* [22], *Thermococcus* *marinus* [23], *Thermococcus* *pacificus* [24],  
208  
209 *Thermococcus* *thioreducens* [25], *Thermococcus* *waiotapuensis* [26], and  
210  
211 *Thermococcus* *barophilus* Ch5 [27]. All these DNA pols have been proven to be used  
212  
213 efficiently in routine PCR.  
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219  
220 The euryarchaeon *Thermococcus gammatolerans* was isolated from a  
221  
222 hydrothermal vent located in the Gulf of California [28]. This euryarchaeon is a  
223  
224 hyperthermophilic archaeon with an optimal growth temperature of 88°C which can  
225  
226 fully withstand a 5.0 kGy dose of gamma irradiation without loss of viability [29], and  
227  
228 thus is the most radioresistant archaeon to date. In reason of its extreme tolerance of  
229  
230 extreme temperatures and irradiation, it was very interesting to test whether the DNA  
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240 polymerases of *T. gammatolerans* has any specific features in comparison to other  
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242 DNA pols from this family. The genome of *T. gammatolerans* encodes two DNA pols,  
243  
244 a Family B DNA pol (Tga PolB) and Family D DNA pol [29]. In this work, we  
245  
246 cloned the Tga PolB gene, expressed its product and characterized its biochemical  
247  
248 characteristics. The recombinant Tga PolB was found capable of bypassing uracil in a  
249  
250 template DNA, which is distinct from other Family B archaeal DNA pols.  
251  
252  
253  
254 Furthermore, biochemical characterization of Tga PolB is discussed.  
255  
256

## 257 **Materials and methods**

### 258 **2.1. Materials**

259  
260 Materials were purchased from the following companies: pET-30a (+), Novagen  
261  
262 (Merck, Darmstadt, Germany); Plasmid Extraction Kit, PCR Cycle Pure Kit, and Gel  
263  
264 Extraction Kit, Omega (Guangzhou, China); *Escherichia coli* DH5 $\alpha$  cells, Transgene  
265  
266 (Beijing, China); dNTPs, T4 DNA ligase, *Nde*I, *Xho*I, and Pfu DNA pol, Thermo  
267  
268 Fisher Scientific (Waltham, MA); Chemicals, Amresco (WA, USA).  
269  
270  
271  
272

### 273 **2.2. DNA substrates**

274  
275 All the oligonucleotides used were synthesized by Sangon Company (Shanghai,  
276  
277 China) and their sequences are listed in Table 1. Primed DNA substrates were  
278  
279 prepared by annealing the Cy3-labeled oligonucleotide with its complementary  
280  
281 oligonucleotides in an annealing buffer containing 20 mM Tris-Cl pH 8.0 and 100  
282  
283 mM NaCl. The annealing reactions were performed at 100°C for 5 min and cooled  
284  
285 slowly to room temperature at least 4 hours.  
286  
287  
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289

### 290 **2.3. Cloning of the Tga PolB encoding gene**

296  
297  
298  
299 The TGAM\_RS07365 gene encoding a Family BDNA pol of *T. gammatolerans*  
300  
301 was amplified by Pfu DNA pol using the genomic DNA as the template in the  
302  
303 presence of the forward primer (5'-GGG AAT TCC ATA TGC  
304  
305 ATTCTCGATACCGACTACATC-3', the italic nucleotides represent *NdeI* restriction  
306  
307 site) and the reverse primer (5'- CCG CTC GAG TCA CTT CTT CCC TTT CAC  
308  
309 CTT CAG-3', the italic nucleotides represent *XhoI* restriction site).The PCR product  
310  
311 was extracted and cleaved by *NdeI* and *XhoI*, and cloned into a pET-30a (+) vector.  
312  
313  
314 The sequence of the recombinant plasmid was verified by sequencing, and the  
315  
316 confirmed plasmid was transformed into *E. coli* BL21 (DE3) RIL cells for expressing  
317  
318 Tga PolB protein with a 6 x His-tag in its C-terminal.  
319  
320  
321

#### 322 323 **2.4. Overexpression and purification of Tga PolB** 324

325 For Tga PolB protein expression, the transformant was cultured at 37°C in LB  
326  
327 medium containing 10 µg/mL kanamycin and 17 µg/mL chloramphenicol until the  
328  
329 OD<sub>600</sub> reached 0.6. Then, isopropyl thiogalactoside (IPTG) was added at a final  
330  
331 concentration of 0.8 mM for 12 hours at room temperature to induce the expression  
332  
333 from the recombinant plasmid.  
334  
335  
336

337 The induced cells were harvested by centrifugation and resuspended in a Ni  
338  
339 column buffer A containing 20 mM Tris-HCl pH 8.0, 1 mM dithiothreitol (DTT), 500  
340  
341 mM NaCl, 50 mM imidazole and 10% glycerol. The cells were immediately disrupted  
342  
343 by ultrasonication at 4°C. After removing cell debris by centrifugation (16,000 g) at  
344  
345 4°C, the supernatant was heated at 70°C for 20 min. The supernatant was then  
346  
347 collected after centrifugation (16,000 g) at 4°C and was loaded onto a HisTrap FF  
348  
349  
350  
351

355  
356  
357  
358 column (GE Healthcare, Uppsala, Sweden). The column was eluted with NCG™  
359  
360 Chromatography System (Bio-Rad, Hercules, CA, USA) using a linear gradient of  
361  
362 50–500 mM imidazole with a Ni column buffer B containing 20 mM Tris-HCl (pH  
363  
364 8.0), 1 mM DTT, 500 mM NaCl, 500 mM imidazole and 10% glycerol. Fractions  
365  
366 containing the His-tagged Tga PolB protein were collected and analyzed by migration  
367  
368 on a 10% SDS-PAGE. The purified Tga PolB protein fractions were dialyzed against  
369  
370 a storage buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 50% glycerol and  
371  
372 50 mM NaCl, and stored at -80°C. The Tga PolB protein concentration was  
373  
374 quantitated by measuring the absorbance at 280 nm. The theoretical molar extinction  
375  
376 coefficient of the enzyme protein is predicted to be 122,300M<sup>-1</sup> cm<sup>-1</sup>.  
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## 382 **2.5. Polymerase assays**

383

384 The standard reaction mixture (10μL) contained 50 nM Tga PolB, 200 nM Cy3-  
385  
386 labeled primed DNA substrate (p22/t59), 20 mM Tris-HCl pH8.0, 2 mM DTT, 100  
387  
388 μg/mL BSA, 2 mM MgCl<sub>2</sub>, and 200 μM dNTPs. The polymerization reactions were  
389  
390 performed for 15 min at 65°C. The polymerized product was analyzed by  
391  
392 electrophoresis in a 15% polyacrylamide gel containing 8 M urea. The gel was  
393  
394 visualized with a Molecular Image analyzer (Bio-Rad). ImageQuant software was  
395  
396 used for quantitative analysis. All polymerase experiments were repeated three times.  
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398  
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400

## 401 **2.6. 3'–5' exonuclease assays**

402

403 The standard reaction mixture (10 μL) contained an indicated amount of Tga  
404  
405 PolB, 200 nM Cy3-labeled ssDNA, 20 mM Tris-HCl pH 8.0, 2 mM DTT, 100 μg/mL  
406  
407 BSA, and 2 mM MgCl<sub>2</sub>. Reactions were carried out for 15 min at 65°C. The cleaved  
408  
409  
410



414 products were subjected to electrophoresis in a polyacrylamide urea gel as described  
415 above. The gel was visualized with a Molecular Image analyzer (Bio-Rad).  
416  
417 ImageQuant software was used for quantitative analysis. All exonuclease experiments  
418  
419 were repeated three times.  
420  
421  
422  
423  
424  
425

## 426 **2.7. Electrophoretic mobility shift assays (EMSA)**

427  
428  
429 200 nM Cy3-labeled ssDNA or primed DNA were incubated with Tga PolB with  
430  
431 varied concentrations in 20 mM Tris-HCl pH 8.0, 1 mM DTT and 8% glycerol at  
432  
433 room temperature for 10 min. The retarded product was analyzed by electrophoresis  
434  
435 in a 4% native polyacrylamide gel in 0.1 x TBE buffer and visualized with a  
436  
437 Molecular Image analyzer (Bio-Rad). ImageQuant software was used for quantitative  
438  
439 analysis. All DNA-binding experiments were repeated three times.  
440  
441  
442

## 443 **2.8. PCR amplification assays**

444  
445  
446 To determine whether Tga PolB can be used in routine PCR, we performed PCR  
447  
448 reactions containing 50 mM Tris-Cl pH8.0, 0.1 mg/ml BSA, 5 mM Mg<sup>2+</sup>, 50 mM  
449  
450 NaCl and 130 nM Tga PolB to amplify the gene TBCH5v1\_0629 (744 bp) using the  
451  
452 genomic DNA of *Thermococcus barophilus* Ch5 as a template and PCR primers  
453  
454 described in our previous publication [30]. The cycling of PCR reactions was  
455  
456 performed as follows: one initial denaturation at 98°C for 5 min followed by 30cycles  
457  
458 of 95°C for 30s, 50 °C 30s and 72 °C1min, and the final step was 72 °C 10 min. PCR  
459  
460 products were resolved by electrophoresis in a 0.8% standard agarose gel.  
461  
462  
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464

## 465 **Results**

### 466 **3.1. Cloning, expression and purification of Tga PolB protein**

473  
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475  
476 The genome of *T. gammatolerans* encodes a Family B DNA polymerase. To  
477  
478 characterize Tga PolB biochemically, its gene was cloned into a pET30a (+)  
479  
480 expression vector, and overexpressed in *E.coli*. The recombinant Tga PolB protein  
481  
482 was purified to near homogeneity after Ni-column affinity purification (Fig. 1A). The  
483  
484 purified protein displayed an approximate MW of 90 kDa (Fig. 1A), which correlates  
485  
486 with the deduced amino acid sequences.  
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488  
489

490  
491 Using the primed DNA (p22/t59), we determined the polymerase activity of the  
492  
493 purified Tga PolB protein using concentrations ranging from 1 to 500 nM. As shown  
494  
495 in Fig.1B, the full length product was gradually created with increasing Tga PolB  
496  
497 concentrations at 65°C, and maximal activity (>90 polymerization percentage) was  
498  
499 obtained at  $\geq 20$  nM, suggesting that the enzyme is able to efficiently synthesize DNA  
500  
501 at high temperature.  
502  
503  
504

505 The genome of *E.coli* encodes five DNA pols that can synthesize DNA, which  
506  
507 might interfere with our above observations [31]. To rule out this possibility, we  
508  
509 prepared the *E.coli* cell extract with the empty pET-30a (+) plasmid as described for  
510  
511 preparation of the recombinant pET30a (+) plasmid with the Tga PolB gene. We used  
512  
513 the cell extract to perform above DNA synthesis reactions, and found that no  
514  
515 synthesized product was observed (data not shown). Thus, the possibility of  
516  
517 contamination of *E.coli* DNA pols was removed, thereby indirectly confirming that  
518  
519 Tga PolB can synthesize DNA at high temperature.  
520  
521  
522

### 523 524 **3.2. Biochemical characterization of Tga PolB**

525  
526 The biochemical characteristics of DNA synthesis by Tga PolB, which includes  
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535 thermostability, the optimal pH, divalent cation availability, salt adaptation and  
536  
537 dNTPs availability, were investigated using the primed DNA described above as the  
538  
539 substrate. Considering the instability of primed duplex used in this work at >65°C, we  
540  
541 performed DNA synthesis reaction of Tga PolB at <65°C. As shown in Fig. S1, the  
542  
543 enzyme is capable of synthesizing DNA at increasing temperatures ranging from 25°C  
544  
545 to 65°C with varied efficiencies High synthesis efficiency (99%) was observed from  
546  
547 45°C to 65°C, further confirming that Tga PolB can replicate DNA at high  
548  
549 temperature.  
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551  
552

553  
554 To investigate its thermo-tolerance, 500 nM Tga PolB was heated at varying  
555  
556 temperatures for different times, and the heated enzyme (50 nM) was used to perform  
557  
558 DNA synthesis reactions at 65°C. Our results showed that the enzyme is able to fully  
559  
560 withstand 95°C for at least 1.0 hr, retaining a  $93 \pm 2.6\%$  activity (Fig. 2A). After 2  
561  
562 hours at 95°C, the enzyme retained ca.  $20 \pm 2.1\%$  activity. Thus, these observations  
563  
564 confirm that, as expected from DNA pols from other *Thermococcus*, Tga PolB is a  
565  
566 thermostable DNA pol.  
567  
568  
569

570  
571 To investigate the effect of pH on Tga PolB activity, we reduced enzyme  
572  
573 concentrations to be 15 nM in DNA synthesis. As shown in Fig. 2B, the enzyme could  
574  
575 synthesize DNA in a broad pH range from 5.0 to 10.0, at which the activity begins to  
576  
577 be altered. No product is synthesized at pH=11. The maximal synthesis activity was  
578  
579 observed between pH 7.0 and 9.0, but our test did not allow having more precision on  
580  
581 the effective optimum.  
582  
583  
584

585  
586 It is well-known that DNA pols utilize a divalent cation to synthesize DNA. To  
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591  
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593  
594 investigate the effect of a divalent cation on Tga PolB activity, we reduced enzyme  
595  
596 concentrations to be 15 nM in DNA synthesis. As expected, no product was observed  
597  
598 in DNA synthesis by Tga PolB without a divalent cation or with EDTA (Fig. 2C).  
599  
600 Only two divalent ions, Mg<sup>2+</sup> and Mn<sup>2+</sup>, could restore DNA pol activity to the enzyme.  
601  
602 The polymerization percentage of Tga PolB was estimated to be 72 ± 5.7% and 33 ±  
603  
604 7.1% in the presence of Mg<sup>2+</sup> and Mn<sup>2+</sup>, respectively, demonstrating that similarly to  
605  
606 other *Thermococcus* DNA PolB, DNA synthesis activity of Tga PolB is dependent on  
607  
608 a divalent cation. Thus, Mg<sup>2+</sup> is the preferred ion for polymerization activity of Tga  
609  
610 PolB.  
611  
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615  
616 We investigated the salt tolerance of the enzyme from 0 to 600 mM by using 15  
617  
618 nM enzyme in DNA synthesis reactions. In absence of NaCl, Tga PolB displayed 67 ±  
619  
620 2.2% polymerization activity (Fig. 2D). From 50 to 200 mM NaCl, the activity was  
621  
622 higher than 90%, but it was significant reduced at NaCl concentrations above 400mM.  
623  
624 Thus, NaCl is required for optimal Tga PolB activity. But, enzyme activity was  
625  
626 inhibited by 400 mM NaCl, and no synthesis was observed at 600 mM NaCl.  
627  
628  
629

### 630 **3.3. Effect of dNTPs on the balance of the polymerase and exonuclease activity of** 631 632 **Tga PolB** 633 634

635  
636 Most of Family B DNA pols, including the members from Archaea, possess 3'-5'  
637  
638 exonuclease activity in addition to 5'-3' polymerase activity. To examine whether Tga  
639  
640 PolB has exonuclease activity, we used ssDNA as a substrate. As expected, we found  
641  
642 that Tga PolB started to degrade DNA when adding 5 nM enzyme to the exonuclease  
643  
644 reaction, and exonuclease activity increased with increasing enzyme concentration  
645  
646  
647  
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649

650  
651  
652  
653 (Fig. 3A). Maximal exonuclease efficiency (>90%) was obtained when for  
654  
655 concentrations of 15 nM Tga PolB and higher. Furthermore, DNA products with  
656  
657 various sizes were created in the presences of varied concentrations of Tga PolB.  
658

659  
660 Next, we investigated the effect of dNTPs on the balance of the polymerase  
661  
662 activity and exonuclease activity of Tga PolB. We designated Exo/Pol as the effect of  
663  
664 dNTPs concentration on the balance of exonuclease activity and polymerase activity.  
665  
666 As the the dNTPs concentrations increased, the values of Exo/Pol decreased,  
667  
668 suggesting that dNTPs concentrations are favor in polymerase activity. At 4.0  $\mu$ M  
669  
670 dNTPs, the values of Exo/Pol reached 0.07 (Fig. 3B), indicating that Tga PolB  
671  
672 exhibits extremely weak exonuclease activity in the presence of 4.0  $\mu$ M dNTPs. Thus,  
673  
674 dNTPs stimulates Tga PolB to synthesize DNA rather than degrade DNA.  
675  
676  
677  
678

### 679 **3.4. Mismatch extension of Tga PolB**

680  
681 We determined mismatch the extension efficiency of Tga PolB by using primed  
682  
683 DNA with a match end (C/G) and primed DNA with a mismatch end (C/A, A/G and  
684  
685 T/G) as substrates. As shown in Fig. 4, no matter what concentration of Tga PolB was  
686  
687 used in polymerase reactions, >95% polymerization percentage was observed during  
688  
689 DNA synthesis by Tga PolB when using the primed DNA with a match as the  
690  
691 substrate. However, Tga PolB displayed only about  $18 \pm 1.5\%$ ,  $15 \pm 2.8\%$  and  $14 \pm$   
692  
693  $4.3\%$  activity for extending A/G, G/G and T/G mismatch at 20 nM, respectively.  
694  
695 Furthermore, at 50 nM, the efficiencies of the enzyme were still lower than for  
696  
697 matched DNA, with  $83 \pm 5.7\%$ ,  $72 \pm 7.8\%$  and  $77 \pm 0.8\%$  for extending A/G, G/G  
698  
699 and T/G mismatch at 50 nM, respectively. Only at 200 nM the enzyme displayed full  
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709  
710  
711  
712 efficiency for extending mismatched DNA. Overall, these results suggested that Tga  
713  
714 PolB has a low efficiency to extend mismatched DNA, although capable to extend it  
715  
716  
717 at high DNA/protein ratios.

### 718 719 **3.5. Bypass activity of uracil and AP in DNA by Tga PolB**

720  
721 Archaeal Family B DNA pols can recognize uracil in DNA, which results in  
722  
723 polymerization arrest. Using uracil-containing primed DNA as the substrate, we tested  
724  
725 this ability in Tga PolB. Note that the full length template DNA is 59 mer and uracil  
726  
727 in the template DNA is located at 42 mer described in Table 1. As shown in Fig. 5,  
728  
729 the full length product was observed at 200 nM Tga PolB in the control reaction using  
730  
731 the normal primed DNA as the substrate. Compared with the control reaction, two  
732  
733 products corresponding to 42 mer (partial length) and 59 mer (full length) were  
734  
735 synthesized by 200 nM Tga PolB using the uracil-containing primed DNA as the  
736  
737 substrate. Furthermore, more synthesized full length product than partial length  
738  
739 product was created by Tga PolB, suggesting that this pol can bypass the uracil of the  
740  
741 DNA template, which is a distinct feature from typical archaeal Family B DNA pols.  
742  
743  
744  
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748  
749 Since Tga PolB could bypass U bases in its DNA template, we tested whether it  
750  
751 could also bypass AP sites in DNA. Like uracil-containing DNA, the AP site in the  
752  
753 full length template DNA (59mer) is also located in 42 mer position. By using AP-  
754  
755 containing primed DNA as a substrate, we conducted DNA synthesis by Tga PolB. As  
756  
757 shown in Fig. 5, only one band corresponding to 42 mer product was observed by Tga  
758  
759 PolB, which is sharply inconsistent with the observation of the replication of uracil  
760  
761 containing DNA by this pol. In contrast, no full length product was synthesized by  
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771 Tga PolB, suggesting that this pol cannot bypass AP sites, as observed in other  
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773 archaeal Family B DNA Pols.  
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### 775 776 **3.6. DNA Binding by Tga PolB** 777

778 Since Tga PolB can degrade ssDNA and extend primed DNA with both matched  
779 and mismatched ends, we employed the EMSA to determine whether Tga PolB is able  
780 to bind DNA. We incubated ssDNA, primed DNA and primed DNA with a mismatch  
781 (T/G) with Tga PolB at varying concentrations, respectively. As shown in Fig. 6A, the  
782 binding of free ssDNA substrate increased gradually with increasing Tga PolB  
783 concentrations. No ssDNA binding was observed at 100 nM and maximal binding  
784 was observed at 500 nM.  
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794 dsDNA binding also increased as Tga PolB concentration increased, from  $49 \pm$   
795  $5.0\%$ ,  $80 \pm 4.8\%$ , and  $86 \pm 3.6\%$  at 100 nM, 200 nM and 300 nM enzyme (Fig. 6B).  
796  
797 At higher concentrations ( $>400$  nM), the binding percentages reached  $>90\%$  and  
798 reached its maximum ( $\sim 97\%$ ) at 500 nM. Thus, Tga PolB has a slightly higher affinity  
799 for primed DNA binding than for ssDNA binding. Tga PolB binding to primed DNA  
800 is not affected by the presence of mismatches (Fig. 6C).  
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### 809 **3.7. PCR amplification of Tga PolB** 810

811 Last, we investigated whether or not Tga PolB can be used for routine PCR to  
812 amplify DNA. Using the genomic DNA of *T. barophilus* Ch5 as a template, we  
813 performed the PCR reaction to amplify the UDG247 gene with Tga PolB. As shown  
814 in Fig. 7, the PCR product was clearly created by Tga PolB, corresponding to 744 bp,  
815 thus suggesting that this Pol can be employed in routine PCR.  
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## Discussion

In this work, we characterized biochemically the Family B DNA pol from the hyperthermophilic and radioresistant euryarchaeon *T. gammatolerans*, and revealed that Tga PolB can replicate DNA at high temperature. Consistent with other *Thermococcus* DNA pol homologues, Tga PolB displays similar biochemical characteristics for DNA synthesis. As expected, Tga PolB can be used to amplify DNA by routine PCR reaction, suggesting that this Pol also resembles other *Thermococcus* DNA pols. Interestingly, Tga PolB has extraordinary properties that are distinct from other archaeal Family B DNA pols.

Crystal structures of archaeal Family B DNA pols demonstrate that they possess uracil recognition pocket [9; 32], thus allowing the replication to be stopped when these DNA pols encounter a uracil in DNA template. Biochemical data provide strong support for the ‘read-ahead’ mechanism. Archaeal B family DNA polymerases, such as Pfu DNA pol, can recognize uracil in DNA template strand during DNA synthesis and will be stalled 4 bp upstream of a template uracil. Thereby preventing G:C→A:T mutation [33]. Although this PolB can bypass uracil in DNA template with low efficiency, the hyperthermoacidophilic crenarchaeon *Sulfolobus solfataricus* DNA polB1 (Sso PolB1) also has similar read-ahead behavior [34]. Furthermore, Pfu PolB displays much weaker ability to bypass uracil in DNA template than Sso PolB1 [34]. However, we demonstrated that Tga PolB can efficiently synthesize DNA despite encountering uracil in DNA template, which differs from other *Thermococcales* enzymes such as Pfu PolB, and Sso PolB1. Consistent with Sso PolB1, Tga PolB



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889 cannot continue to replicate DNA when encountering an AP lesion in DNA template,  
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891 consistent with other archaeal Family B DNA pols [34; 35].  
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894 As DNA polymerase substrate, deoxynucleotide triphosphates affect the  
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896 exonuclease/polymerase balance of a DNA polymerase. It has been reported that  
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898 dNTPs stimulate polymerase activity of a DNA polymerase and suppress its  
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900 exonuclease activity. However, different DNA polymerases display distinct sensitivity  
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902 to the dNTP inhibition. In this study, we demonstrate that Tga PolB is capable of  
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904 extending primer at a low level (2  $\mu$ M) of dNTPs, which is consistent with Sso polB1  
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906 [36]. In contrast to Sso PolB1, which retains its exonuclease activity up to 600  $\mu$ M  
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908 dNTPs, Tga PolB already lacks an exonuclease activity at 5  $\mu$ M dNTPs [36].  
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913 A replicative DNA polymerase has polymerase activity and 3'-5' exonuclease  
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915 activity, and is in favor of exonuclease activity when encountering a mismatched  
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917 primer end to ensure high fidelity of DNA replication. In this study, we show that Tga  
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919 PolB is able to efficiently extend primer with a correct pair end at 20 nM. However,  
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921 Tga PolB displays significantly reduced extension efficiencies for primers with  
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923 uncorrected pair ends at 20 nM, which may be caused by the exonuclease activity of  
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925 Tga PolB that can remove the mismatched 3' nucleotide to generate a match base pair.  
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927 Thus, Tga PolB might be in favorable for the exonuclease activity for removing  
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929 uncorrected nucleotide when encountering a mismatched pair end and then start to  
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931 synthesize DNA after proofreading.  
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936 Last, we also show that Tga PolB displays higher affinity for binding primed  
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938 DNA than for binding ssDNA at <300 nM. Furthermore, two bands corresponding to  
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948 primed DNA binding appeared while only band corresponding to ssDNA binding of  
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950 was observed at both 200 nM and 300 nM Tga PolB. We proposed that one primed  
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952 DNA molecular would be bound with two Tga PolB molecular whereas one Tga PolB  
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954 molecular might bind one ssDNA molecular. Overall, Tga PolB has distinct binding  
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956 patterns for primed DNA and ssDNA.  
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### 959 960 **Conclusion**

961  
962 In summary, we present the biochemical characteristics of the Family B DNA  
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964 pol from the hyperthermophilic and radioresistant *T. gammatolerans*. We demonstrate  
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966 that the recombinant Tga PolB possesses polymerase activity for DNA synthesis and  
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968 exonuclease activity for removing misincorporated nucleotide. Surprisingly, Tga PolB  
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970 can replicate DNA when encountering uracil in DNA template, which is sharply  
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972 contrasted with other archaeal Family B DNA pols. However, this pol can be stalled  
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974 by an AP site in DNA. Furthermore, dNTPs stimulate the polymerase activity and  
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976 inhibit the exonuclease activity of Tga PolB. Tga PolB can bind to primed DNA with  
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978 higher efficiencies than to ssDNA at low concentration, and also display distinct  
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980 binding patterns for primed DNA and ssDNA. Last, Tga PolB can be used in routine  
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982 PCR.  
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993  
994

### 995 **Author contributions**

996  
997 LZ and ZY designed experiments; DJ, HS, MW and QG performed experiments;  
998  
999

1004  
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1006  
1007 LZ, ZY, and PO analyzed data; LZ, ZY, and PO wrote and revised the paper.  
1008  
1009

### 1010 **Conflict of interests**

1011  
1012 All authors declare that there is no conflict of interests regarding the publication  
1013  
1014 of this paper.  
1015

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### 1035 **Figure legends**

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1038 **Fig. 1.** Tga PolB is able to synthesize DNA at 65°C. A. the overexpression and  
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1040 purification of the recombinant Tga PolB protein. B. DNA synthesis of Tga PolB at  
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1042 65°C. The DNA synthesis reactions were performed by Tga PolB at 65°C in the  
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1044 presence of varied concentrations. The synthesized DNA products were analyzed by  
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1046 running a denaturing polyacrylamide gel. CK: the reaction without the enzyme.  
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1051 **Fig. 2.** Biochemical characterization of Tga PolB. A. the thermo-tolerance of the  
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1053 enzyme; B. the optimal pH of the enzyme activity; C. the effects of divalent cations  
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1055 on the enzyme activity; D. the effect of NaCl on the enzyme activity. The DNA  
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1057 synthesis reactions were performed at 65°C. The synthesized DNA products were  
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1066 analyzed by a denaturing polyacrylamide gel. CK in panels A, B and D, and CK1 in  
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1068 panels C: the reaction without the enzyme; CK2 in panel C: the reaction without a  
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1070  
1071 divalent cation.

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1073 **Fig. 3.** Inhibition of exonuclease activity of Tga PolB by dNTPs substrate. A. the  
1074  
1075 exonuclease activity of Tga PolB. Tga PolB with varied concentrations was employed  
1076  
1077 to perform exonuclease reactions by using the ssDNA as the substrate at 65°C. The  
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1079 cleaved DNA products were analyzed by a denaturing polyacrylamide gel. B. the  
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1081 effect of dNTPs substrate on the polymerase and exonuclease activities of Tga PolB.  
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1083 The coupled polymerase and exonuclease activities of Tga PolB were performed at  
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1085 65°C in the presence of dNTPs with varied concentrations. The reaction products  
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1087 were analyzed by a denaturing polyacrylamide gel. CK: the reaction without the  
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1089 enzyme.  
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1095 **Fig. 4.** Mismatch extension by Tga PolB. The match DNA (C:G) and mismatch DNA  
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1097 (A:G, G:G and T:G) were employed as the substrates in the DNA synthesis reactions  
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1099 catalyzed by Tga PolB (20, 50 and 200 nM) at 65°C. The synthesized DNA products  
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1101 were analyzed by running a denaturing polyacrylamide gel. CK: the reaction without  
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1103 the enzyme.  
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1107 **Fig. 5.** Bypass analysis of uracil and AP in DNA by Tga PolB. The uracil- and AP-  
1108  
1109 containing primed DNA were employed as the substrates in the DNA synthesis  
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1111 reactions catalyzed by 200 nM Tga PolB at 65°C. The synthesized DNA products  
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1113 were analyzed by running a denaturing polyacrylamide gel. CK: the reaction without  
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1115 the enzyme.  
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1125 **Fig. 6.** DNA binding assays of Tga PolB. 200 nM ssDNA and primed DNA were  
1126  
1127 incubated with Tga PolB with varied concentrations at room temperature, respectively.  
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1129 The retarded DNA products were analyzed by a native polyacrylamide gel. A. binding  
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1131 ssDNA; B. binding normal primed DNA; C. binding primed DNA with a mismatch  
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1133 (T/G). CK: the binding reaction without the enzyme.  
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1137 **Fig. 7.** PCR amplification of Tga PolB. M: DNA marker; CK: the PCR reaction  
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1139 without enzyme.  
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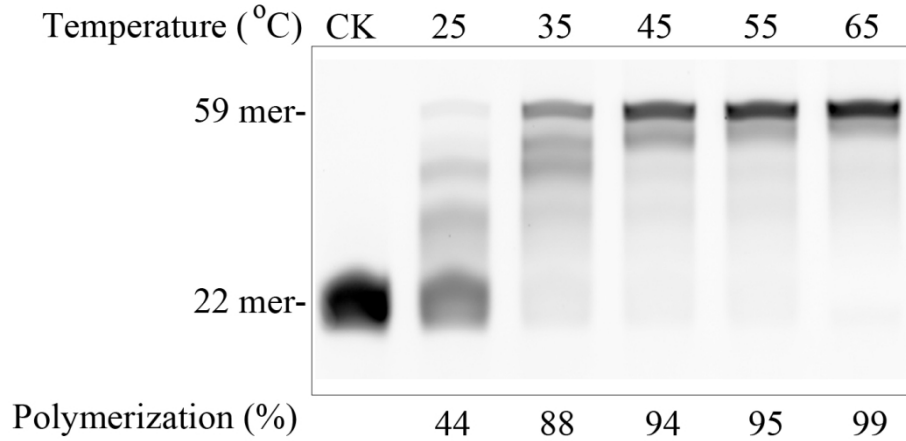
**Table 1** Sequences of the oligonucleotides used in this work

Name	Sequence (5'-3')
p22C*	CAG TGA ATT CGA GCT CGG TAC C
p22A*	CAG TGA ATT CGA GCT CGG TAC A
p22G*	CAG TGA ATT CGA GCT CGG TAC G
p22T*	CAG TGA ATT CGA GCT CGG TAC T
t59	GCT TGC ATG CCT GCA GGT CGA CTC TAG AGG ATC CCC GGG TAC CGA GCT CGA ATT CAC TG
t59U	GC TTG CAT GCC TGC AGG <u>UCGA</u> CTC TAG AGG ATC CCC GGG TAC CGA GCT CGA ATT CAC TG
t59AP	GCT TGC ATG CCT GCA <u>GGAPCGA</u> CTC TAG AGG ATC CCC GGG TAC CGA GCT CGA ATT CAC TG

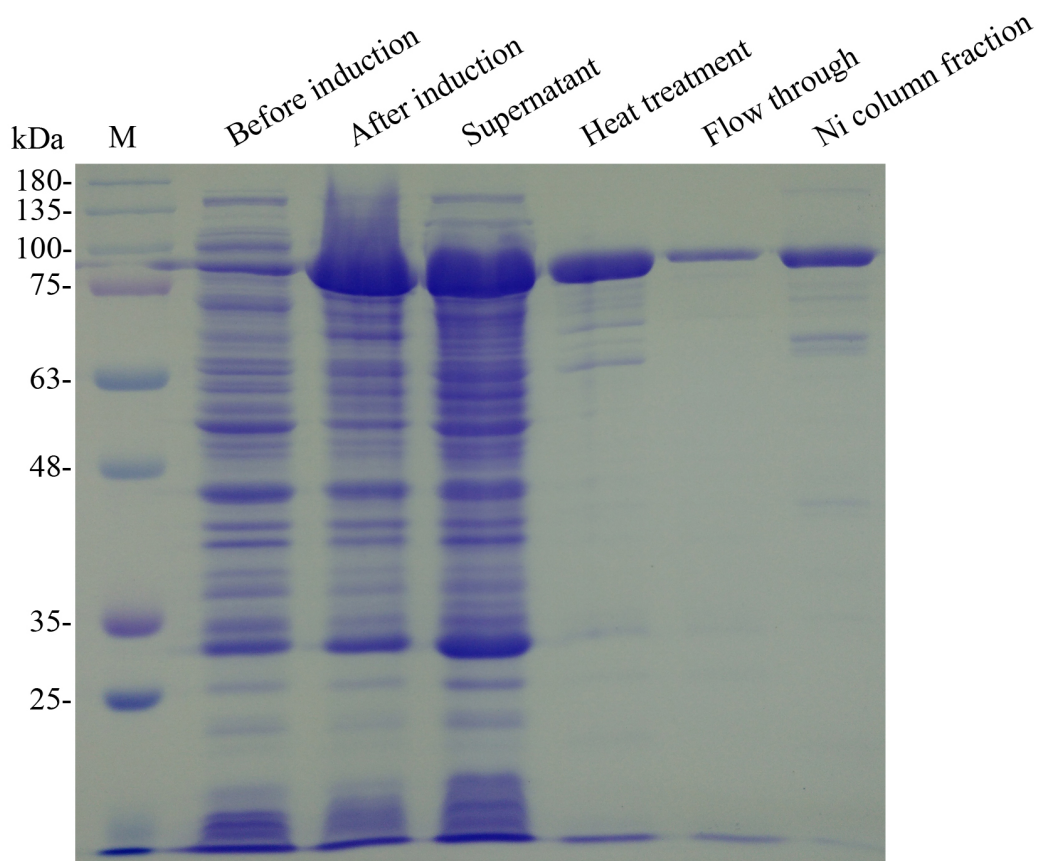
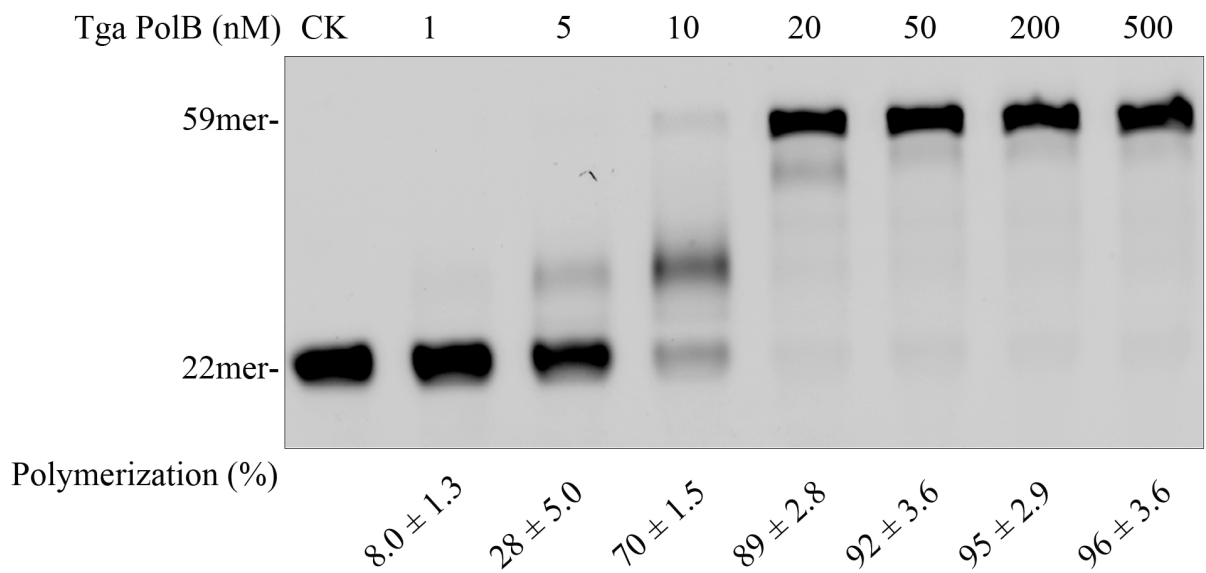
\*indicates the Cy3-labeling at 5' end of primers (p22C, p22A, p22G and p22T).

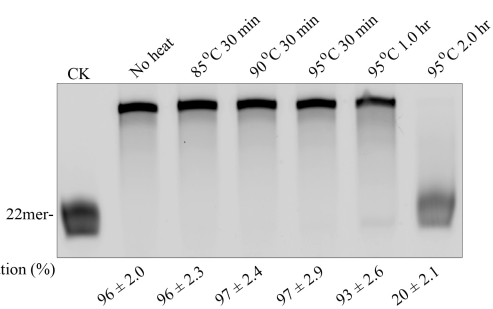
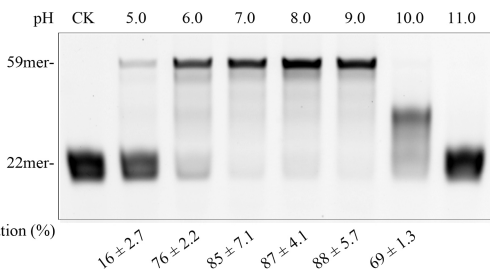
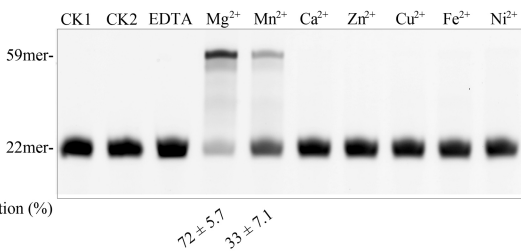
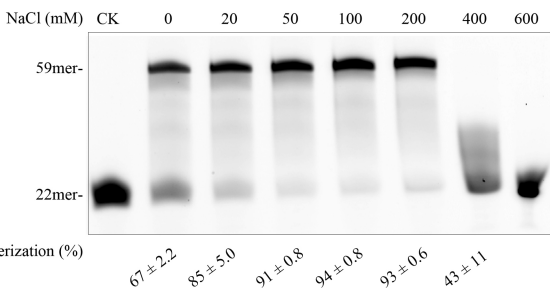
The underlined base is used to prepare uracil (U)- and AP (Apurinc/aprimidinic)-containing dsDNA.

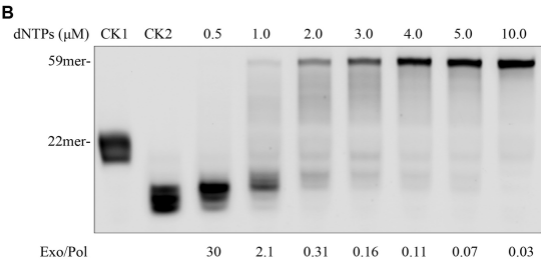
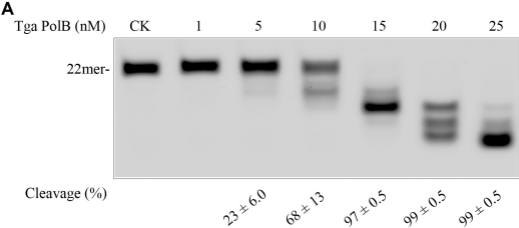
1594  
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1600 **Supplemental Data**  
1601  
1602  
1603



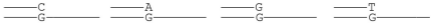
1620 **Fig. s1.** DNA synthesis of Tga PolB at different temperatures. The DNA synthesis  
1621 reactions were performed by 50 nM Tga PolB at different temperatures. The  
1622 synthesized DNA products were analyzed by running a denaturing polyacrylamide gel.  
1623  
1624  
1625  
1626  
1627 CK: the reaction without the enzyme.  
1628  
1629  
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1631  
1632  
1633  
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1649

**A****B**

**A****B****C****D**



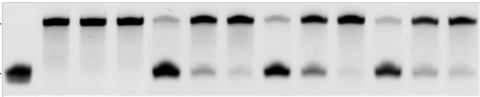




Tga PolB (nM) CK 20 50 200 20 50 200 20 50 200 20 50 200

59 mer-

22 mer-



Polymerization (%)

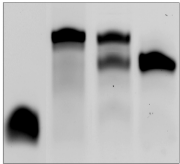
$95 \pm 5.0$ 
 $96 \pm 3.6$ 
 $98 \pm 1.3$ 
 $18 \pm 1.5$ 
 $83 \pm 5.7$ 
 $93 \pm 4.1$ 
 $15 \pm 2.8$ 
 $72 \pm 7.8$ 
 $96 \pm 4.0$ 
 $14 \pm 4.3$ 
 $77 \pm 0.8$ 
 $94 \pm 9.2$

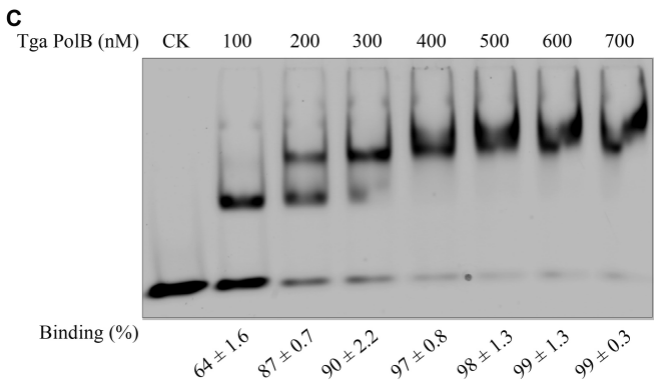
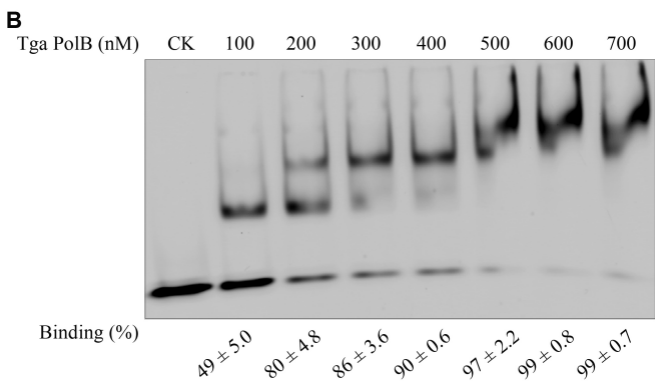
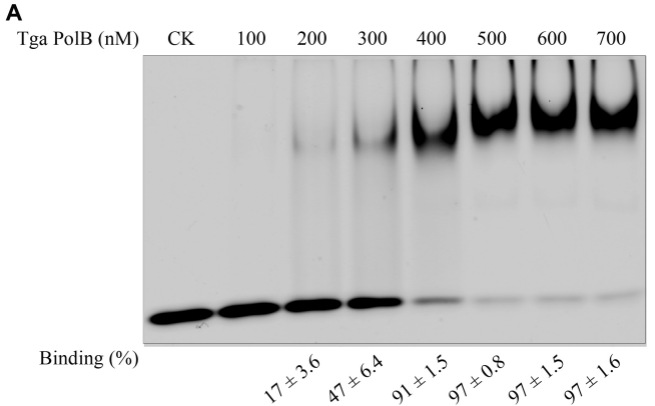
CK      Normal DNA      U-containing DNA      AP-containing DNA

59mer-

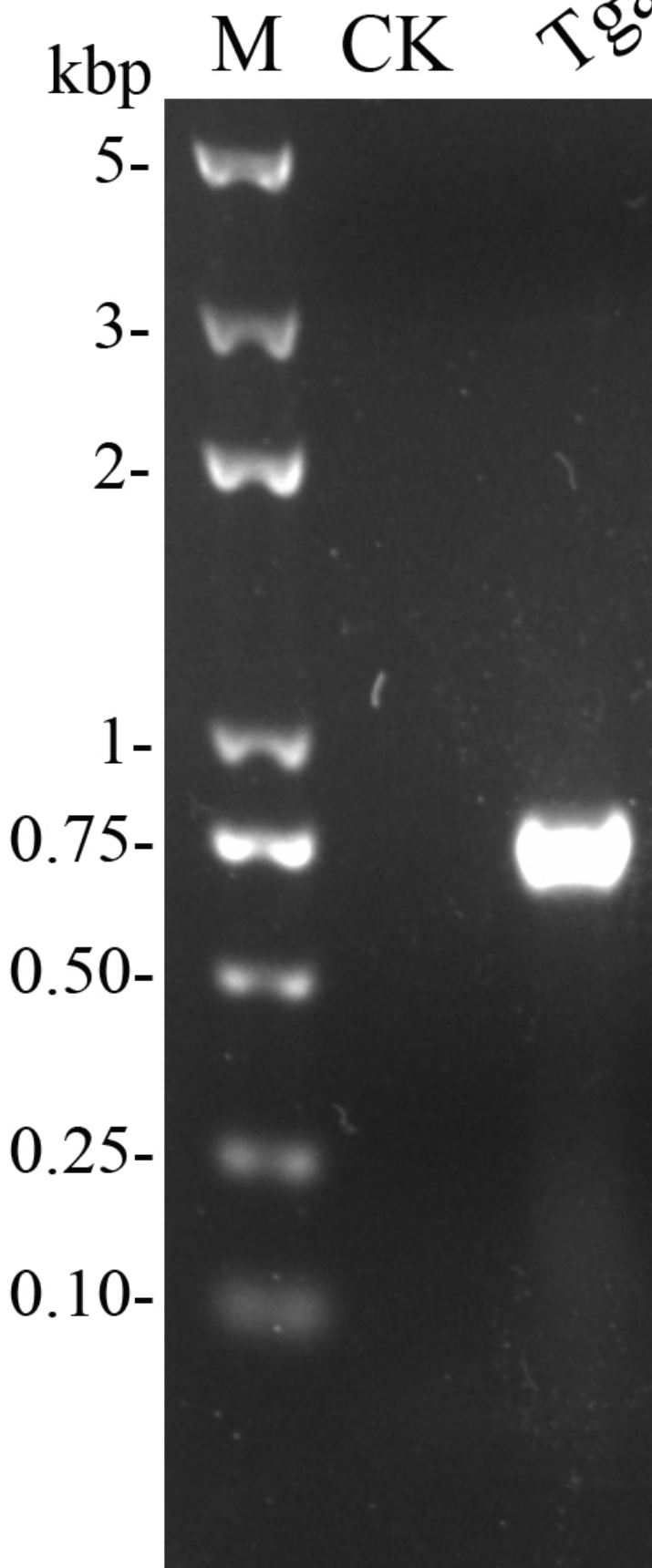
42mer-

22mer-





Tga PolB



## Author Statement

All authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

## SupplementalData

**Characterization and application of a Family B DNA polymerase from the  
hyperthermophilic and radioresistant euryarchaeon *Thermococcus*  
*gammatolerans***

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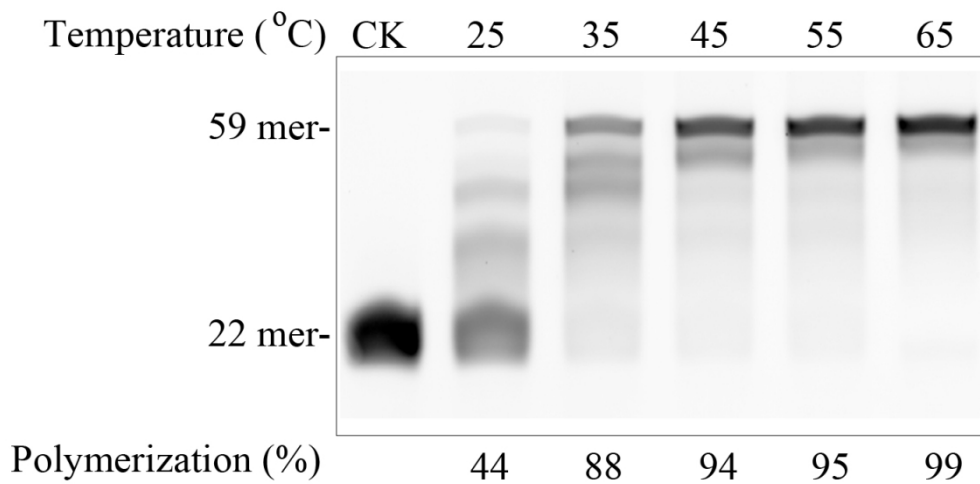
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**Fig. s1.** DNA synthesis of Tga PolB at different temperatures. The DNA synthesis reactions were performed by 50 nM Tga PolB at different temperatures. The synthesized DNA products were analyzed by running a denaturing polyacrylamide gel. CK: the reaction without the enzyme.