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Cloning, Purification, and Characterization of a New DNA Polymerase from a Hyperthermophilic Archaeon, *Thermococcus* sp. NA1

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Abstract Genomic analysis of *Thermococcus* sp. NA1 revealed the presence of a 3,927-base-pair (bp) family B-type DNA polymerase gene, *TNA1_pol*. TNA1_pol, without its intein, was overexpressed in *Escherichia coli*, purified using metal affinity chromatography, and characterized. TNA1_pol activity was optimal at pH 7.5 and 75°C. TNA1_pol was highly thermostable, with a half-life of 3.5 h at 100°C and 12.5 h at 95°C. Polymerase chain reaction parameters of TNA1_pol such as error-rate, processivity, and extension rate were measured in comparison with *rTaq*, *Pfu*, and KOD DNA polymerases. TNA1_pol averaged one incorrect bp every 4.45 kilobases (kb), and had a processivity of 150 nucleotides (nt) and an extension rate of 60 bases/s. Thus, TNA1_pol has a much faster elongation rate than *Pfu* DNA polymerase with 7-fold higher fidelity than that of *rTaq*.

Keywords: Hyperthermophile, characterization, family B-type DNA polymerase, processivity, fidelity

Recent advances in genomic research *via* the combination of conventional genetic engineering and genomic research techniques have produced vast amounts of sequence information. The genomic sequences of hyperthermophilic microorganisms are of considerable biotechnological interest because they are natural sources of heat-stable enzymes that can be developed for biotechnological purposes.

Polymerase chain reaction (PCR), which uses a thermostable DNA polymerase, is one of the most important developments in protein and genetic research [19] and is currently used in a broad array of biological applications. More than 50 DNA polymerase genes have been cloned from various organisms, including thermophiles and archaea [9, 16, 21]. Family B-type DNA polymerases from two hyperthermophilic archaea, *Pyrococcus* and *Thermococcus*, have enjoyed recent popularity for use in PCR, because they offer higher fidelity than the *Taq* DNA polymerase commonly used based on their proofreading activities [11, 13, 14, 22, 23]; however, there is demand for the improvement of high-fidelity enzymes because of their low elongation rates [2].

We isolated a new hyperthermophilic strain from a deep-sea hydrothermal vent area in the PACMANUS field. It was identified as a species of *Thermococcus* based on its 16S rDNA sequence [1], and whole-genome sequencing is underway to search for thermostable enzymes. Analysis of the genome indicated that the strain possessed a family B-type DNA polymerase. In this study, the DNA polymerase gene was cloned and expressed in *Escherichia coli*, and the enzymatic characteristics of the purified recombinant enzyme were examined.

Materials and Methods

Strains and Culture Conditions

Thermococcus sp. NA1 was isolated from a deep-sea hydrothermal vent area in the East Manus Basin of the PACMANUS field (3°14' S, 151°42' E). YPS medium [8] was used to culture the archaeon for DNA manipulation; culture and strain maintenance were performed according to standard procedures [18]. To prepare a seed culture of *Thermococcus* sp. NA1, YPS medium in a 25-ml serum bottle was inoculated with a single colony from a phytagel plate and cultured at 85°C for 20 h. Seed cultures were used to inoculate 700 ml of YPS medium in an anaerobic jar and cultured at 85°C for 20 h. *E. coli* strain DH5α was used for plasmid propagation and nucleotide sequencing. *E. coli* strain BL21-CodonPlus(DE3)-RIL cells (Stratagene, LaJolla, CA, U.S.A.) and the plasmid pET-24a(+) (Novagen,

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Madison, WI, U.S.A.) were used for gene expression. *E. coli* strains were cultivated in Luria-Bertani medium with 50 μg/ml kanamycin at 37°C.

DNA Manipulation and Sequencing

DNA manipulations were performed using standard procedures, as described by Sambrook and Russell [20]. Genomic DNA of *Thermococcus* sp. NA1 was isolated using a standard procedure [18]. Restriction enzymes and other modifying enzymes were purchased from Promega (Madison, WI, U.S.A.). Small-scale preparation of plasmid DNA from *E. coli* cells was performed using a plasmid mini-prep kit (Qiagen, Hilden, Germany). DNA sequencing was performed using an ABI3100 automated sequencer, using a BigDye terminator kit (PE Applied Biosystems, Foster City, CA, U.S.A.).

Cloning and Expression of the *TNA1_pol* Gene

The polymerase-coding region of *TNA1_pol* was constructed by overlapping PCR [6]. The regions encoding the N-terminal portion (sense [5'-CGACCCGG*CATATG*ATCCT CGACGTCGATTACATCACAG-3'] and antisense [5'-GCCGTAGTACCCGTAATAGCTGT TCGCTAAGATTT-TTATTGCCCGCTG-3']) and the C-terminal portion (sense [5'-CAGCGGGCAATAAAAATCTTAGCGAACAGCT-ATTACGGGTACTACGGC-3'] and antisense [5'-CTCCA-CAT*CTCGAG*TTTCTTCGGCTTCAACCAAGCC CC-3']) of TNA1_pol were amplified separately using primers containing the overlapping sequences. The full-length *TNA1_pol* gene flanked by NdeI and XhoI sites was then amplified by PCR using two primers (sense [5'-CGACCC-GG*CATATG*ATCCTCGACGTCGATTACATCACAG-3'] and antisense [5'-CTCCACAT*CTCGAG*TTTCTTCGGCT-TCAACCAAGCCCC-3']; the italicized sequences indicate the NdeI site in the sense primer and the XhoI site in the antisense primer) and the mixture of N-terminal and C-terminal PCR fragments as templates. The amplified fragment was digested with NdeI and XhoI and ligated into NdeI/XhoI-digested pET-24a(+). The ligation product was then transformed into *E. coli* DH5α cells. Positive transformants were selected using restriction enzyme digestion, and the clones were confirmed by DNA sequencing.

Purification of TNA1_pol

The expression plasmid was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL cells, and overexpression of the *TNA1_pol* gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase, followed by a 3-h incubation at 37°C. The cells were harvested by centrifugation (6,000 ×*g* at 4°C for 20 min) and resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol. The cells were disrupted by sonication; after centrifugation (20,000 ×*g* at 4°C for 30 min), a crude enzyme sample was prepared by heat treatment at 80°C for 20 min. The resulting supernatant was applied to a column of TALON metal affinity resin (BD Biosciences) and washed with 10 mM imidazole (Sigma) in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol; TNA1_pol was eluted in the same buffer with 300 mM imidazole. The pooled fractions were dialyzed into storage buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, and 10% glycerol.

The protein concentration was determined by Bradford [3] assay, and protein purity was examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using standard procedures [12].

DNA Polymerase Activity Assay

DNA polymerase activity was measured as described by Choi and Kwon [4], with slight modifications. The enzyme was incubated at 75°C for 10 min in a 25-μl reaction mixture consisting of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 100 μM each dATP, dCTP, and dGTP, 0.25 μCi of [methyl-^{3}H]thymidine 5'-triphosphate, and 625 ng of activated calf-thymus DNA (Promega). An aliquot was spotted onto a DE81 filter paper disc (23 mm; Whatman, U.K.), and the disc was dried on a heat block. The disc was washed first with 0.5 M sodium phosphate buffer (pH 7.0) for 10 min, then with 70% ethanol for 5 min, and dried. The incorporated radioactivity was counted using a Beckman LS6500 scintillation counter (Fullerton, CA, U.S.A.). One unit of enzymatic activity was defined as the amount of DNA polymerase that incorporated 1 pmol of [^{3}H]TTP into an acid-insoluble product at 75°C in 10 min.

Exonuclease Activity Assay

Exonuclease activity was measured using 3' end-labeled DNA and 5' end-labeled DNA as substrates. In brief, pBluescript SK plasmid, linearized by NotI, was filled in by Klenow fragment in the presence of [α-^{32}P]dCTP, and a 2-kb PCR product was phosphorylated by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. After labeling, the DNA substrates were purified by ethanol precipitation and incubated with the enzyme in a 25-μl reaction mixture consisting of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 20 mM $(NH_4)_2SO_4$, and 0.01% bovine serum albumin at 75°C for 10 min in the presence or absence of dNTPs. The reaction was precipitated by adding 1 ml of 5% trichloroacetic acid in the presence of BSA as a carrier. After centrifugation, the supernatant was withdrawn and its radioactivity was counted using a Beckman LS6500 scintillation counter.

PCR-Fidelity Assay

The error rate of TNA1_pol during PCR was determined by direct sequencing. A 2-kb target from λ DNA was amplified using either 1.3 units (U) of TNA1_pol or 2.5 U

Table 1. Primers used in this study.

Primer name	Primer sequence
Primers used for fidelity assays	
L-F	5'-CCTGCTC TGCCGCTTCACGC-3'
L-2R	5'-CCATGATTCAGTGTGCCCGTCTGG-3'
Primers used for processivity and extension-rate assays	
Hex-labeled M13 oligomer	5'-Hex-CCGTTGCTACCCTCGTTCCGATGC-3'
M13 oligomer	5'-CCGTTGCTACCCTCGTTCCGATGC-3'
Primers used for target length efficiency assays	
G-F	5'-ACTAAATTGGTGATACCGTTATGAG-3'
G-2R	5'-GGAACATAAAATGTAAGGGACTTC-3'
G-4R	5'-GTCTCTGATGCTCATGATGTAGTTC-3'
G-8R	5'-GAGGAGCTCTTTAGAATTCTCAAGC-3'
L-5R	5'-CGAACGTCGCGCAGAGAAACAGG-3'
L-8R	5'-GCCTCGTTGCGTTTGTTTGCACG-3'
L-10R	5'-GCACAGAAGCTATTATGCGTCCCCAGG-3'
L-12R	5'-TCTTCCTCGTGCATCGAGCTATTCGG-3'
L-15R	5'-CTTGTTCCTTTGCCGCGAGAATGG-3'

of *rTaq* (Takara) DNA polymerase (Table 1). The PCR products were cloned into pCRII-TOPO (Invitrogen) and transformed into *E. coli* DH5α. Fifty clones from each reaction were randomly selected, and the fragments of interest were sequenced. The error rate was calculated as the ratio of the number of errors to the total nucleotides read.

Processivity Assay

A 5' Hex-labeled M13-primer (400 fmol; Table 1) was added to M13mp18 ssDNA (200 fmol) in a reaction mixture containing 20 mM Tris-HCl (pH 8.5), 1 mM $MgCl_2$, 60 mM KCl, 30 mM $(NH_4)_2SO_4$, and 0.2 mM dNTPs. The mixture was preheated at 95°C for 1 min and incubated at 62°C for 1 min using a T1 thermocycler (Biometra). Finally, TNA1_pol was added to the mixture, which was incubated at 75°C for 10 sec. The resulting DNA fragments were analyzed using an ABI3100 automated sequencer. To ensure that no multiple binding/extension occurred on any primer-template complex, both the polymerase concentration and the reaction time were varied and the median product length was determined for each reaction [25]. When the median length no longer changed with an increase in reaction time or a decrease in polymerase concentration, the traces of those samples were used to determine the enzyme's processivity using the method of Von Hippel *et al.* [24]. The processivity of *Pfu* (Promega), *rTaq* (Takara), and KOD (Novagen) DNA polymerases was similarly determined using each manufacturer's buffer.

Extension-Rate Assay

The extension rate was determined from the product length synthesized during a fixed reaction time. M13mp18 ssDNA and M13 primers were used as template and primers, respectively (Table 1). A 40-μl reaction mixture containing 0.8 μg of M13mp18 ssDNA, 8 pmol of M13 primer, 0.2 mM each of dATP, dGTP, and dTTP, 0.05 mM dCTP, 0.05 mM [α-^{32}P]dCTP (18.5 MBq), 2.5 U of DNA polymerase, and the optimized buffer for each polymerase was incubated at 75°C. The TNA1_pol buffer consisted of 20 mM Tris-HCl (pH 8.5), 1 mM $MgCl_2$, 60 mM KCl, 30 mM $(NH_4)_2SO_4$, and 0.1% Triton X-100. Use of the *Pfu* (Promega), *rTaq* (Takara), and KOD (Novagen) DNA polymerases followed the manufacturer's instructions. After incubation for the indicated time, an equal volume of stop solution consisting of 60 mM EDTA and 60 mM NaOH was added. A 10-μl aliquot of each sample was analyzed using agarose gel electrophoresis followed by autoradiography.

Long and Accurate (LA) PCR

To test the performance of TNA1_pol in amplifying a broad range of target DNA, genomic or λ DNA was used as a template. Primers (Table 1) were designed to amplify fragments 2, 5, 8, 10, 12, and 15 kb in length from λ DNA and 2, 4, and 8 kb in length from *Thermococcus* sp. NA1 (DQ223723) genomic DNA. Each PCR reaction was carried out in a 50-μl mixture containing 50 ng of template DNA, 20 pmol of primers, 350 μM dNTPs, and PCR reaction buffer. The PCR buffer consisted of 20 mM Tris-HCl (pH 8.5), 30 mM $(NH_4)_2SO_4$, 60 mM KCl, and 1 mM $MgCl_2$. After a single 5-min denaturation step at 95°C, 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C), and extension (1 min/kb at 72°C) were performed, followed by a final 7-min extension at 72°C. PCR products were analyzed using 0.8% agarose gel electrophoresis.

Nucleotide Sequence Accession Number

The nucleotide sequence of *TNA_pol* was submitted to the GenBank/EMBL Data Bank with Accession No. DQ223721.

RESULTS

Cloning and Expression of *TNA1_pol*

We isolated a hyperthermophilic archaeon, *Thermococcus* sp. NA1, growing at 70–90°C, and determined its entire genomic sequence. Analysis of the genomic sequence revealed a 3,927-bp open reading frame with similarity to family B-type DNA polymerases. The gene contained a putative 3'–5' exonuclease domain, an α-like DNA polymerase domain, and one in-frame intervening sequence of 1,605 bp (535 amino acid intein) located in the middle of the PolIII region. The splicing sites of the intein coding sequence were predicted as reported [7, 17], and the size of *TNA1_pol* alone was estimated as 2,322 bp, encoding a protein consisting of 773 amino acid residues (Fig. 1). In a pairwise alignment with other DNA polymerases, the deduced amino acid sequence of TNA1_pol showed 91.0% identity with KOD DNA polymerase (Accession No. D29671), 82.0% identity with Deep-vent DNA polymerase, and 79.0% identity with *Pfu* DNA polymerase (Accession No. D12983). To

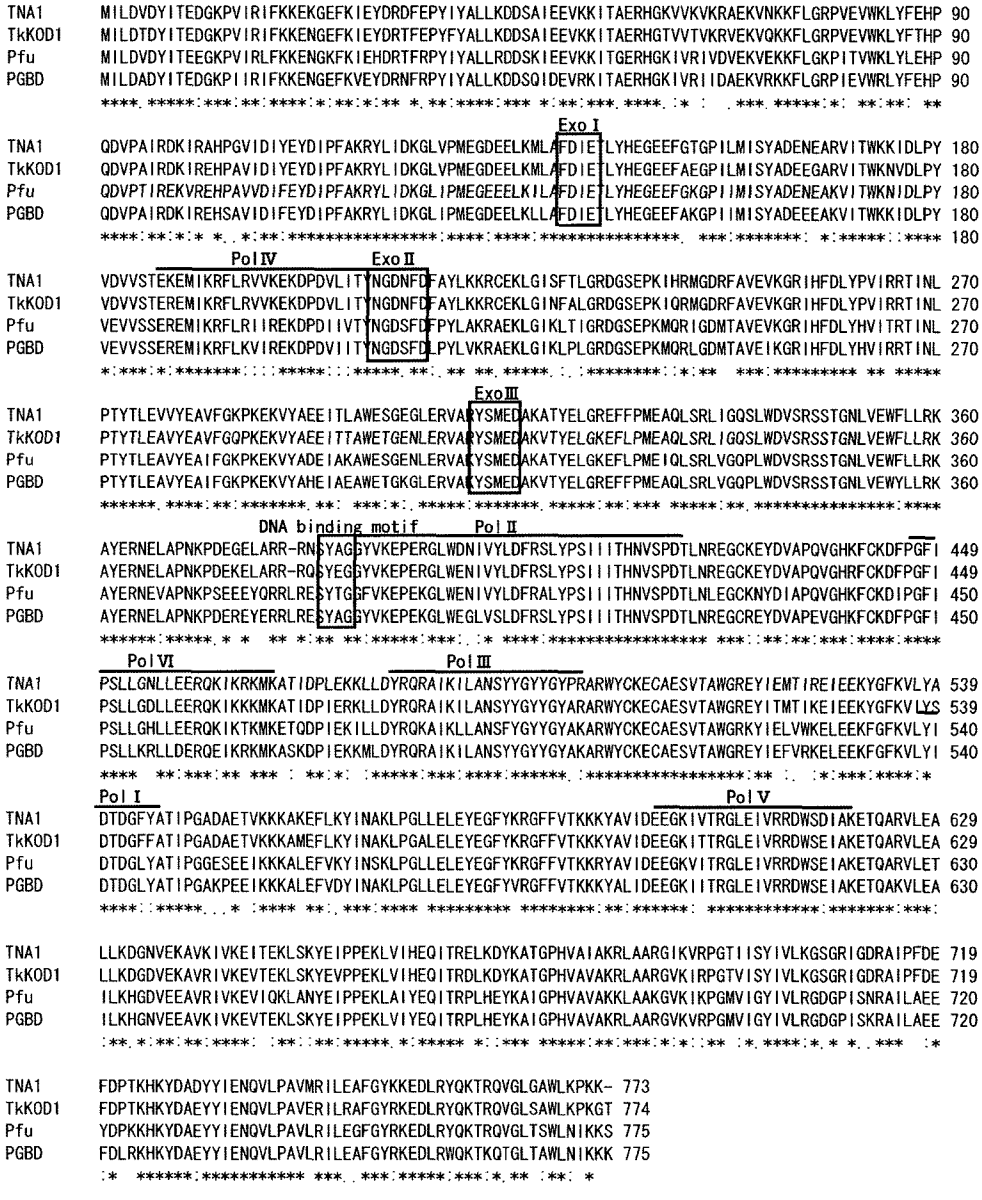

Fig. 1. Sequence comparison of family B-type DNA polymerases from *Thermococcus* sp. NA1 (TNA1), *T. kodakarensis* KOD1 (TkKOD1, GenBank Accession No. D29671), *Pyrococcus furiosus* (Pfu, DDBJ Accession No. D12983), and *Pyrococcus* sp. GB-D (PGBD, GenBank Accession No. U00707).

Dashes indicate gaps and the numbers at the right represent the position of the last residue in the original sequence. Identical residues among the four enzymes are marked with asterisks (*) and residues with conserved substitutions and semiconserved substitutions are marked with colons (:) and and periods (.), respectively. The polymerase domain (PolI through PolVI), exonuclease domain (ExoI through ExoIII), and the DNA-binding motif are marked.

produce TNA1_pol for PCR, a construct containing the coding form of *TNA1_pol* in pET-24a(+) was expressed in *E. coli*.

Characterization of TNA1_pol

The recombinant TNA1_pol was soluble and was purified using TALON metal affinity chromatography. SDS-PAGE revealed a major protein band with a molecular mass of 90 kDa (Fig. 2). The purified protein remained soluble after repeated freezing and thawing cycles.

An activity assay using activated calf-thymus DNA as a template revealed that the DNA polymerase functioned optimally at pH 7.5 and 75°C. TNA1_pol was thermostable, with a half-life ($t_{1/2}$) of 3.5 h at 100°C and 12.5 h at 95°C (data not shown).

The DNA polymerase was highly dependent on $MgCl_2$, with maximal activity at 6 mM $MgCl_2$ and no detectable activity in the absence of $MgCl_2$. $(NH_4)_2SO_4$ also affected the activity of TNA1_pol, with an optimal concentration of 20 mM. The polymerase was not greatly affected by variations in KCl concentration, however, and was resistant to KCl concentrations above 100 mM (data not shown).

The *TNA1_pol* gene (Fig. 1) contained putative 3'→5' exonuclease domains (ExoI, ExoII, and ExoIII), indicating that TNA1_pol may have 3'→5' exonuclease activity. To address this possibility, the 3'→5' and 5'→3' exonuclease activities of TNA1_pol were measured. TNA1_pol could release about 68% of the ^{32}P from 3' end-labeled DNA within 1 h, whereas the amount of ^{32}P released from 5' end-labeled DNA was very low and did not increase without dNTPs (Fig. 3). This demonstrates that TNA1_pol possesses 3'→5' exonuclease activity, as do most archaeal family B-type DNA polymerases [10], but no 5'→3' exonuclease activity.

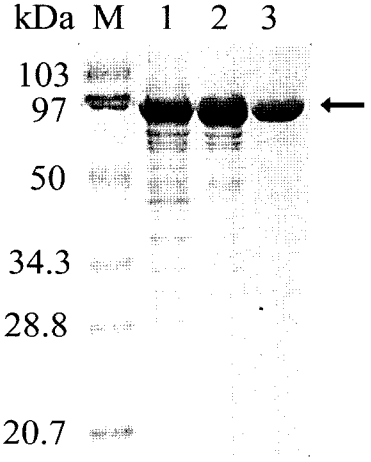

Fig. 2. SDS-PAGE analysis of TNA1_pol. M, standard; 1, crude extract; 2, after heat treatment; 3, purified by His-tagged affinity chromatography.
The molecular mass standards (lane M) include phosphorylase *b* (103 kDa), bovine serum albumin (97 kDa), ovalbumin (50 kDa), carbonic anhydrase (34.3 kDa), soybean trypsin inhibitor (28.8 kDa), and lysozyme (20.7 kDa).

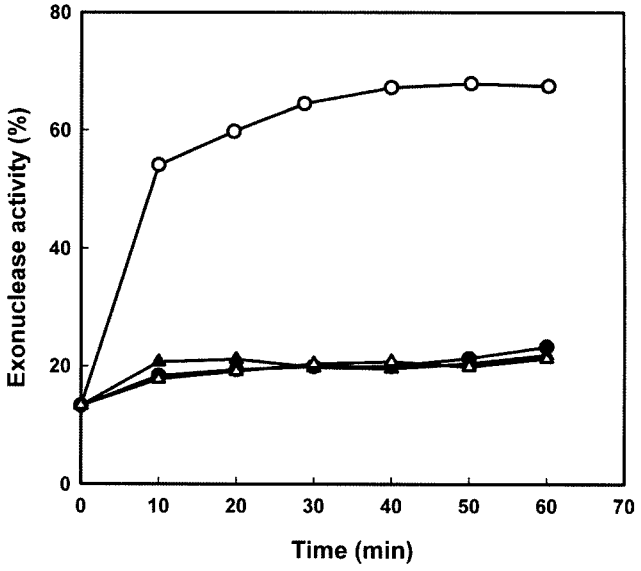

Fig. 3. Measurement of recombinant TNA1_pol activity.
3'→5' exonuclease activity was measured in the absence (○) or presence (●) of dNTPs. 5'→3' exonuclease activity was measured in the absence (△) or presence (▲) of dNTPs. Activity was calculated as the amount of supernatant radioactivity/total radioactivity.

PCR Parameters of TNA1_pol

To evaluate the performance of TNA1_pol during PCR amplification, various parameters were investigated and compared with *rTaq*, *Pfu*, and KOD DNA polymerases. As expected, based on its exonuclease activity, TNA1_pol showed a sevenfold lower error rate than *rTaq* DNA polymerase, introducing an average of one incorrect bp every 4.45 kb (Table 2). The processivity of TNA1_pol was determined as 150 nt, which was twofold greater than that of *Pfu* DNA polymerase (80 nt) and comparable to that of *rTaq* DNA polymerase (160 nt; Fig. 4). Nonetheless, KOD DNA polymerase showed the best processivity (270 nt). The extension rate of TNA1_pol was measured as 60 bases/s, which was 2.5-fold greater than that of *Pfu* DNA polymerase (Fig. 5A), and comparable to *rTaq* DNA polymerase (80 bases/s). The extension rate of TNA1_pol was confirmed by repeated amplifications using varying extension times (Fig. 5B). Taken together, the extension rate and processivity of TNA1_pol were similar to those of *rTaq* and much higher than those of *Pfu*.

Application in LA PCR

It has been reported that DNA polymerases with high fidelity are not suitable for long and accurate (LA) PCR because of their strong exonuclease activity [2]. The maximum PCR product length of TNA1_pol was determined using λ DNA or genomic DNA of *Thermococcus* sp. NA1 as template in a PCR performed as described (see Materials and Methods). TNA1_pol could amplify DNA fragments of archaeal genomic DNA up to 8 kb long, although the

Table 2. Comparison of error rates between TNA1_pol and *rTaq* DNA polymerases.

DNA polymerase	TNA1_pol	*rTaq*
Total reading size (nt)	35,668	43,898
Mismatch (nt)	7	57
Insertion or deletion (nt)	1	14
Error rate*	2.2×10^{-4}	1.6×10^{-3}
Error frequency (1/nt)	1/4,458	1/618

*Error rate was calculated as erroneous nt/total nt.

yield of the 8-kb product was much less than that of the 4-kb and 2-kb products (Fig. 6). Furthermore, it was observed that TNA1_pol could produce a 15-kb-long product from λ DNA. This seems to be a unique feature of TNA1_pol because high-fidelity enzymes such as KOD could not efficiently amplify DNA fragments greater than 8 kb. The fact that TNA1_pol was capable of amplifying DNA fragments up to 15 kb expands its usefulness to include LA PCR.

DISCUSSION

In general, family B-type DNA polymerases from hyperthermophilic archaea have 3'→5' exonuclease activity and offer high-fidelity DNA amplification, but most exhibit poor elongation activity compared with bacterial family A-type DNA polymerases. This has been overcome by the optimization of reaction buffers, the construction of mutants with decreased 3'→5' exonuclease activity, and mixtures of an exonuclease-free DNA polymerase and a proofreading-capable DNA polymerase [15]. Screening for new DNA polymerases or modified DNA polymerases can be an alternative. In this study, the processivity and extension rate of TNA1_pol were similar to those of *rTaq* and much better than those of *Pfu*, although the processivity was still lower than that of KOD DNA polymerase. The reason why TNA1_pol displayed relatively high processivity could be rationalized by comparing its sequence with its homologs. Hashimoto *et al.* [5] claimed that several arginine residues at the forked point of KOD DNA polymerase may be

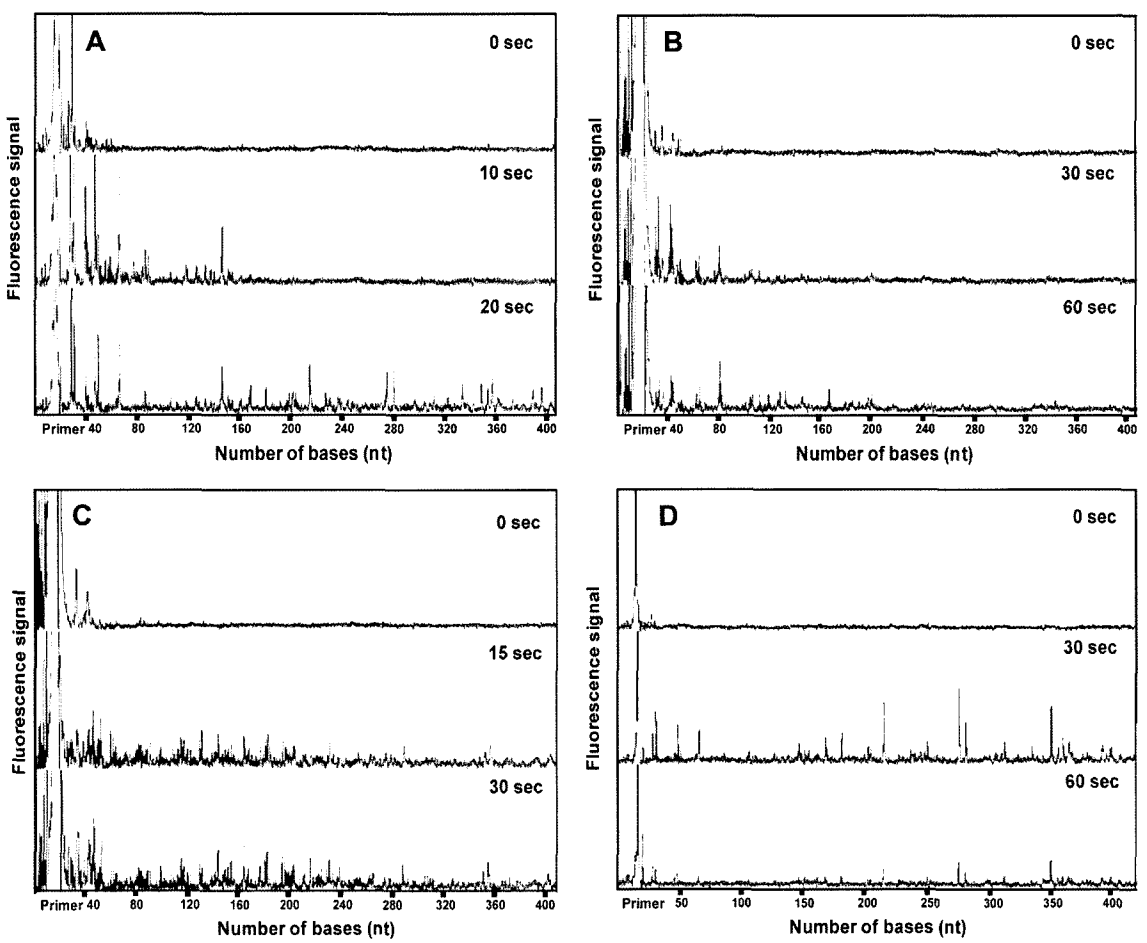

Fig. 4. Comparison of the processivity of TNA1_pol, *Pfu*, *rTaq*, and KOD DNA polymerases.
Each trace represents one lane from a sequencing gel; each peak represents a single primer extension product. Electropherogram traces of (**A**) TNA1_pol, (**B**) *Pfu*, (**C**) *rTaq*, and (**D**) KOD1 DNA polymerases. The labels on the x-axis indicate the product length, determined by size markers run on the same gel.

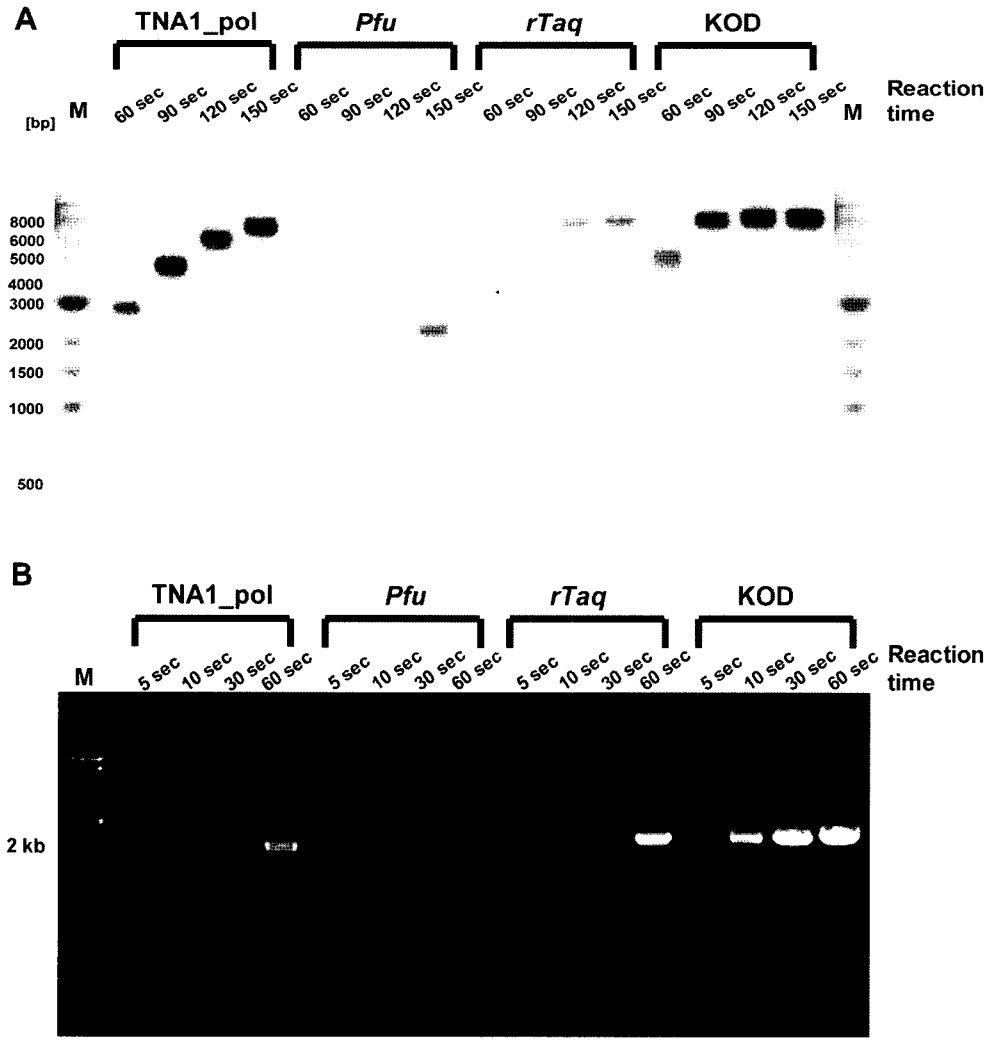

Fig. 5. A. Comparison of the extension rates of TNA1_pol, *Pfu*, *rTaq*, and KOD DNA polymerases. Reaction times are indicated at the top. **B.** Comparison of PCR elongation rates for TNA1_pol, *Pfu*, *rTaq*, and KOD. Elongation times used for PCR amplification are indicated at the top.
Lane M, DNA molecular size marker.

involved in stabilizing the melted structure of DNA strands, contributing to the high processivity of the enzyme. The arginine residues (R243, R247, R265, R266, R346, R381, and R501) identified at the forked point of KOD DNA polymerase are conserved in TNA1_pol. Furthermore, basic residues that face into the KOD DNA polymerase active site at the "Fingers" subdomain are also conserved, except for an R476K substitution. Nevertheless, the difference in processivity between TNA1_pol and KOD DNA polymerase suggests that there is an additional factor regulating processivity. On the other hand, TNA1_pol exhibited some unique features, such as resistance to high salt concentrations and amplification of long DNA targets from λ DNA. Taken together, TNA1_pol seems to have advantages over *Pfu* DNA polymerase in elongation rate and provides a valuable tool for understanding the mechanism of

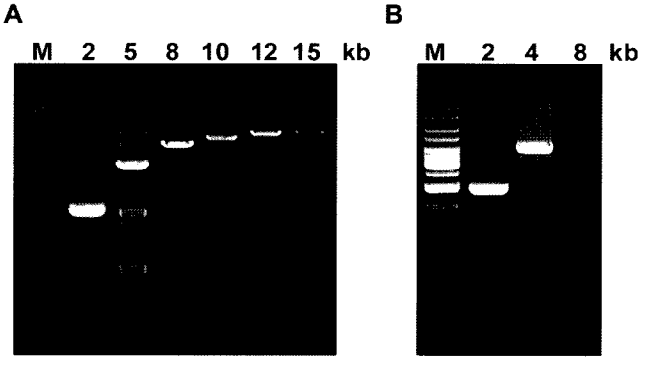

Fig. 6. A. Application of TNA1_pol in LA PCR using λ DNA as a template. M, DNA molecular size marker; target lengths of 2 kb, 5 kb, 8 kb, 10 kb, 12 kb, and 15 kb were marked. **B.** PCR amplification of DNA targets from genomic DNA. M, DNA molecular size marker; target lengths of 2 kb, 4 kb, and 8 kb were marked.

processivity. Further investigation to understand these features is in progress.

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