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# Family B DNA polymerase from a hyperthermophilic archaeon *Pyrobaculum calidifontis*: Cloning, characterization and PCR application

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**The 2352 bp gene coding for 783 amino acid family B DNA polymerase from *Pyrobaculum calidifontis* was cloned and expressed in *Escherichia coli*. Expression of the gene resulted in the production of *Pca*-Pol in soluble fraction. After heat denaturation of the host proteins, the *Pca*-Pol was further purified by ion exchange and hydrophobic interaction chromatographies. Activity gel analysis showed the presence of a catalytically active polypeptide of about 90 kDa. The mass of the protein, determined by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry was found to be 89,156 Da. The isoelectric point of the enzyme was found to be 6.13. The optimal pH and magnesium ion concentration for the enzyme activity were 8.5 and 4 mM, respectively. Unlike other commercially available DNA polymerases the enzyme activity of *Pca*-Pol was inhibited by monovalent cations such as ammonium and potassium. The half-life of the polymerase at 95°C and 100°C was 4.5 h and 0.5 h, respectively. The enzyme possessed 3′→5′ exonuclease activity and was able to amplify, under suitable conditions, up to 7.5 kb DNA fragments by polymerase chain reaction which makes it a potential candidate for amplification of long DNA fragments.**



[**Key words:** Archaea; DNA polymerase; Hyperthermophile; *Pyrobaculum calidifontis*; Exonuclease activity; Polymerase chain reaction]

The domain archaea constitutes the third major branch of living organisms (1). Archaea are divided into four phyla (2,3) namely Crenarchaeota, Euryarchaeota, Korarchaeota and Nanoarchaeota. Many of the crenarchaeotes appear to be hyperthermophiles (organisms growing optimally at 80°C or above). Because of their heat tolerance, proteins from hyperthermophiles are of significant importance (4).

DNA directed DNA polymerase (E.C. number 2.7.7.7) plays an important role in cellular DNA replication and repair. Based on amino acid sequence homology, DNA polymerases have been classified into families A, B, C, D, E, X and Y (5–8). Archaea are known to possess family B and D DNA polymerases (9). The members of crenarchaeota possess family B DNA polymerases (10) whereas euryarchaeotes contain both family B and D DNA polymerases (11,12).

Thermostable DNA polymerases are required in polymerase chain reaction (PCR). PCR is an important method used for DNA amplification, sequencing, diagnosis and site directed mutagenesis (13). DNA polymerase from *Thermus aquaticus* was the first thermostable DNA polymerase to be characterized (14). However, it has no 3′→5′ exonuclease activity which is responsible for the proofreading ability of the enzyme. Archaeal DNA polymerases are known to possess 3′→5′ exonuclease activity and hence have better fidelity as compared to that of *Taq* DNA polymerase.

*Pyrobaculum calidifontis* is a hyperthermophilic archaeon isolated from hot water spring in the Philippines. *P. calidifontis* is a rod shaped, heterotrophic crenarchaeote that grows optimally at 90°C to 95°C and pH 7 in atmospheric air (15).

In this study we describe gene cloning and expression of a thermostable family B DNA polymerase from *P. calidifontis*. The recombinant enzyme was purified and various biochemical properties were studied. Application of the polymerase in PCR was also demonstrated.

## MATERIALS AND METHODS

**Strains, enzymes, vectors, chemicals** *P. calidifontis*, used as the donor organism for DNA polymerase gene, was isolated by our group at Kyoto University, Japan (15). *Escherichia coli* DH5α, *E. coli* BL21 CodonPlus(DE3)-RIL and expression vector pET-21a(+) were from Novagen (Madison, USA), TA cloning vector pTZ57R/T, all enzymes, deoxyribonucleoside triphosphates (dNTPs), dideoxyribonucleoside triphosphates (ddNTPs), biotin chromogenic detection kit, gel extraction kit and GeneJet plasmid isolation kit were purchased from Fermentas (Ontario, Canada). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). Sephadex G-25 spin columns were from GE Healthcare (Piscataway, NJ, USA). TTP [methyl-$^3$H] was from MP Biomedicals (Solon, OH, USA). IPG strips from Serva (Reno, NV, USA) were used for determination of *pI*. Agarose, aphidicolin and activated calf thymus DNA were from Sigma (St. Louis, MO, USA). Oligos (unlabelled and biotinylated) were synthesized commercially by GeneLink (Hawthorne, NY, USA). Medium components for growth of *P. calidifontis* were from Nacalai Tesque Inc. (Kyoto, Japan).

**Growth of *P. calidifontis* and genomic DNA isolation** Growth medium for *P. calidifontis* contained 1% tryptone, 0.1% yeast extract and 0.3% sodium thiosulfate. Glycerol stock of *P. calidifontis* was inoculated (1%) in 25 ml of medium and grown for 24 hours at 90°C with shaking at 100 rpm. This pre-culture was used to inoculate 200 ml growth medium. After growth of two days under same conditions, cells were harvested by centrifugation at 6500 rpm for 15 min at 4°C. Genomic DNA isolation and other DNA manipulations were done using standard procedures (16).

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**PCR amplification and cloning of the polymerase gene** Genomic DNA of *P. calidifontis* was used as a template for PCR amplification of DNA polymerase gene (*Pca*-pol). Genome sequence revealed the presence of two family B DNA polymerases, one belonging to each subfamily B1 and B3. Based on the sequence of the polymerase gene of family B3 (accession no. CP000561), two primers Pol2N (5′-CATATGAGGTTTTGGCCTCTAGACGCCACGTACTCTG-3′) and Pol2C (5′-GCAGAAACTAGCCTAGGAAGTCCAAGAGTG-3′) were synthesized. The forward primer Pol2N contained an *Nde*I recognition site (shown underlined). PCR was performed by using *Taq* DNA polymerase. The PCR conditions were: initial denaturation step at 95°C for 2 min; followed by 30 cycles of: denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 90 s; and a final extension step at 72°C for 10 min. Electrophoresis of the PCR product was carried out and the amplified product was eluted from gel. This gel purified DNA polymerase gene was ligated to TA cloning vector pTZ57R/T (Fermentas) and transformed *E. coli* DH5α. This recombinant plasmid was named pol-pTZ57. This plasmid was then digested with *Nde*I and *Bam*HI to liberate the polymerase gene which was gel purified and ligated to pET-21a(+) (Novagen) digested with the same restriction enzymes. The resulting recombinant expression vector was named pol-pET21a.

**DNA sequencing and sequence analysis** After cloning in expression vector, the presence of *Pca*-pol was confirmed by DNA sequencing using CEQ800 Beckman Coulter sequencing system. Multiple sequence alignment was performed by using ClustalW programme (17).

**Expression of *Pca*-pol gene and purification of recombinant protein** *E. coli* BL21 CodonPlus(DE3)-RIL cells were transformed with expression vector pol-pET21a and grown in LB medium. Gene expression was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) at an $OD_{600}$ of 0.4, followed by further incubation for 4 h at 37°C with shaking at 100 rpm. Cells were harvested by centrifugation at 6500 rpm for 15 min at 4°C. The cell pallet was suspended in 25 mM Tris–Cl pH 8.5 and lysed by sonication. After centrifugation at 15,000 rpm for 15 min at 4°C, the supernatant obtained was heated at 80°C for 30 min and centrifuged. Nucleic acids were precipitated by addition of polyethylenimine (0.3% final concentration) followed by centrifugation. The resulting supernatant was subjected to anion exchange chromatography by using ÄKTApurifier FPLC system (GE Healthcare). The anion exchange column (ResourceQ-6 ml) was equilibrated with 25 mM Tris–Cl pH 8.5 and the crude enzyme preparation was loaded onto the column. Proteins were eluted by a linear gradient of 0 to 1 M NaCl in equilibration buffer. Major fractions containing the *Pca*-Pol were collected and dialyzed against 25 mM Tris–Cl pH 8.5. After dialysis, $(NH_4)_2SO_4$ was added to protein solution (1 M final concentration) and loaded onto hydrophobic interaction column (ResourcePHE-1 ml) equilibrated with 25 mM Tris–Cl pH 8.5 containing 1 M $(NH_4)_2SO_4$. *Pca*-Pol was eluted by a linear gradient of 1 to 0 M $(NH_4)_2SO_4$ prepared in 25 mM Tris–Cl pH 8.5. The fractions containing *Pca*-Pol were dialyzed against the storage buffer (25 mM Tris–Cl pH 8.5, 0.1% Tween 20, 0.2 mg/ml BSA and 50% glycerol) and stored at −20°C.

**DNA polymerase activity gel analysis** DNA polymerase activity gel analysis was performed as described in literature by Kahler and Antranikian (18) with slight modifications. All buffers contained 25 mM Tris–Cl pH 8.5, $MgCl_2$ was used at a concentration of 4 mM, the labeled nucleotide was biotin-11-UTP and 2U purified *Pca*-Pol was used for activity gel analysis. *Taq* DNA polymerase and Klenow fragment (1U each) were used as positive and negative controls, respectively. After transfer of DNA to Hybond N+ membrane (GE Healthcare), detection of incorporated biotin-11-UTP was done by using biotin chromogenic detection kit (Fermentas) according to the instructions of the manufacturer.

**Determination of molecular mass and isoelectric point** Molecular mass analysis of the recombinant protein by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) was performed. The purified enzyme was desalted by Sephadex G-25 column (GE Healthcare). 1 µl of salt free protein solution (5 mg/ml) was mixed with 10 µl of 3,5-dimethoxy-4-hydroxycinnamic acid (10 mg/ml) prepared in 1/3 of acetonitrile, 2/3 of 0.1% trifluoroacetic acid (TFA) in water and 1 µl was applied to target plate (Bruker, USA). The sample was allowed to dry at room temperature for 15–20 min. Spectrum was obtained by striking 3000 shots in an acquisition mass range of 70,000–100,000 Da. Final spectrum was subjected to smoothing, baseline subtraction and centroiding.

Isoelectric focusing was done for determination of isoelectric point of *Pca*-Pol. SERVA precast IPG strip of linear pH gradient from pH 4 to 7 was used. Electrophoresis was performed on 2D-IEF-SYS (Scie-Plas, UK).

**Assay for DNA polymerase activity** DNA polymerase activity was monitored by measuring incorporation of TTP [methyl-$^3$H] in activated calf thymus DNA. The reaction mixture, in 20 µl, contained: 25 mM Tris–Cl pH 8.5, 4 mM $MgCl_2$, 100 µM each of dATP, dGTP, dCTP, dTTP, 0.5 µCi TTP [methyl-$^3$H] (85 Ci/mmol), 5 µg activated calf thymus DNA, 0.2 mg/ml BSA and 0.1% Tween 20. The mixture was preincubated at 75°C for 5 min before the addition of the enzyme. After the addition of the enzyme, aliquots were removed from the reaction mixture at various time intervals and spotted onto DE-81 filter paper discs (23 mm diameter, Whatman, Madison, UK). The discs were dried, washed three times in sodium phosphate buffer pH 7.0 followed by washing in 70% ethanol (2 min for each wash) and dried. The incorporated radioactivity on the dried filter discs was measured in counts per second (cps) by using Raytest Malisa scintillation counter (Berlin, Germany). One unit of DNA polymerase was defined as the amount of the enzyme required to incorporate 10 nmol [$^3$H]TTP into a polynucleotide fraction (adsorbed on DE-81 filter disc) at 75°C in 30 min.

The effect of concentration of dNTPs on the polymerase activity was analyzed by using biotin labeled primer-template. A 40-mer primer (5′-biotin-CGCACCGTGACGCCAAGCTTGCATTCCTACAGGTCGACTC-3′) annealed to an 80-mer template (5′-biotin-CGTTGCTGACAAACGGGCCGGTCAACAATCCTCTGGAGTCGACCTGTAGGAATGCAAGCTTGGCGTCACGGTGCGCCAAC-3′) was incubated with 0.2U *Pca*-Pol for 5 min at 75°C either in the absence or presence of various concentrations of dNTPs in buffer containing 25 mM Tris–Cl pH 8.5, 4 mM $MgCl_2$, 0.2 mg/ml BSA and 0.1% Tween 20. Reactions were stopped by addition of formamide buffer and incubation on ice. DNA was resolved on 15% polyacrylamide gel in the presence of 7 M urea. After transfer of DNA to Hybond N+ membrane (GE Healthcare), formation of 75-mer product was detected by using biotin chromogenic detection kit (Fermentas).

**Effect of inhibitors** The effect of aphidicolin and dideoxythymidine triphosphate (ddTTP) on the activity of *Pca*-Pol was investigated. Enzyme activity was measured as described above except for analyzing the effect of ddTTP where the reaction mixture did not contain dTTP. In addition to other components, the reaction mixtures contained the indicated concentration of the inhibitor. Assay was performed in the presence of either aphidicolin (0–1000 µM) or ddTTP (ddTTP:TTP [methyl-$^3$H] 0–10). In order to investigate the effect of ddTTP, the concentration of TTP [methyl-$^3$H] was kept constant in all the reaction mixtures while that of ddTTP was varied to achieve a required concentration ratio.

**Assay for exonuclease activity** *Pca*-Pol was also analyzed for the presence of associated 3′→5′ exonuclease activity. In order to prepare 3′ labeled DNA substrate, *Hin*dIII digested λ phage DNA was filled in by exonuclease deficient Klenow fragment in the presence of 100 µM each of dATP, dGTP, dCTP and 1 µCi TTP [methyl-$^3$H]. After labeling, the substrate DNA was purified by sephadex G 25 spin column (GE healthcare). 3′→5′ exonuclease activity was assayed in a 50 µl reaction mixture containing 25 mM Tris–Cl pH 8.5, 4 mM $MgCl_2$ and the labeled substrate DNA. The reaction mixture was preincubated at 75°C for 5 min before the addition of the enzyme. After the addition of the enzyme, aliquots were removed from the reaction mixture at various time intervals and spotted onto DE-81 filter paper discs. The discs were washed and radioactivity was measured as described above. Exonuclease activity was monitored by measuring decrease in radioactivity bound to filter disc. This value was used to calculate the percentage of radioactivity released from labeled substrate DNA.

Analysis of 3′→5′ exonuclease activity was also done by monitoring the degradation of a 40-mer primer annealed to an 80-mer template (described above). The annealed primer-template was incubated with 0.5U of either *Pca*-Pol or *Pfu* or *Taq* DNA polymerases at 75°C for 1 h. For single strand dependent exonuclease assay, only 40-mer was used. After incubation, DNA was resolved on 15% polyacrylamide gel in the presence of 7 M urea, transferred to Hybond N+ membrane (GE Healthcare) and detected by using biotin chromogenic detection kit (Fermentas).

**PCR with purified *Pca*-Pol** To evaluate the extent of amplification produced by the enzyme, PCR amplification of DNA fragments of various lengths was performed. DNA fragments cloned in either pTZ57R/T or pET-22b were used as templates in PCR. Various lengths of amplified products were obtained by using different sets of specific primers. In addition to the respective primer-template set, the PCR mixture in 20 µl contained 10 mM Tris–Cl pH 8.5, 4 mM $MgCl_2$, 200 µM each dNTP, 0.2 mg/ml BSA and 2.5 U *Pca*-Pol. PCR conditions were: initial denaturation at 95°C for 2 min; followed by 30 cycles of: denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C (45 seconds per kb); and final extension at 72°C for 10 min. The entire sample, after PCR, was analyzed by agarose gel electrophoresis.

## RESULTS

**Gene cloning and sequence analysis** A 2352 bp *Pca*-pol open reading frame (ORF) coding for 783 amino acid protein was cloned in pTZ57R/T followed by cloning in expression vector pET-21a(+). Restriction site of *Nde*I was introduced in the forward primer Pol2N which changed the start codon GTG (coding for valine) to ATG (coding for methionine). After cloning, gene sequence was determined and deduced amino acid sequence of the encoded protein was elucidated.

The deduced amino acid sequence of the *Pca*-Pol was aligned and compared to those of other archaeal family B DNA polymerases (Fig. S1). Amino acid sequence alignment revealed the presence of conserved 3′→5′ exonuclease motifs (19) and 5′→3′ polymerase motifs (5). In pairwise alignment, *Pca*-Pol showed 78% identity to *Pyrobaculum islandicum* DNA polymerase (accession no. AF195019), 54% to *Pyrodictium occultum* DNA polymerase (PolB accession no. D38574), 51% to *Aeropyrum pernix* DNA polymerase (Pol II accession no. AB017501), 35% to *Pyrococcus furiosus* DNA polymerase (accession no. D12983) and 30% to *Sulfolobus solfataricus* DNA polymerase (accession no. Y08257).

**Production and purification of *Pca*-Pol** *E. coli* BL21 CodonPlus (DE3)-RIL cells harboring pol-pET21a expression vector were grown in LB medium and gene expression was induced with IPTG. The cells

were harvested, suspended in 25 mM Tris–Cl pH 8.5 and lysed by sonication. After lysis and heating at 80°C for 30 min, *Pca*-Pol was found in soluble fraction and was purified to near homogeneity by anion exchange and hydrophobic interaction chromatographies. Approximately 2 mg of purified product was obtained per liter of *E. coli* culture. The purification process is summarized in Table 1. The specific activity of the purified enzyme was 1133 U/mg with a recovery of 29%. SDS-PAGE analysis of the purified enzyme showed a single band of approximately 90 kDa (Fig. 1) which is in good agreement to the theoretically calculated mass of 89,156 Da based on the amino acid sequence. Activity gel analysis demonstrated that the purified recombinant protein possessed DNA polymerase activity (Fig. S2). In case of *Pca*-Pol, incorporation of biotin-11-UTP was observed at a position corresponding to a mass of about 90 kDa (Fig. S2, lane 1). *Taq* DNA polymerase, a positive control, was able to incorporate biotin-11-UTP (Fig. S2, lane 3) whereas no incorporation could be observed in case of Klenow fragment (Fig. S2, lane 2).

**Determination of molecular mass, isoelectric point and N-terminal sequence** Molecular mass of the purified recombinant *Pca*-Pol was analyzed by MALDI-TOF MS. The molecular mass was found to be 89,156 Da (Fig. S3). Isoelectric point of the enzyme was determined to be 6.13 (data not shown). N-terminal sequence of first five amino acids of the purified protein was determined commercially (Alta Bioscience, Birmingham, UK) which corresponded to the deduced amino acid sequence. These results indicated that the purified protein was *Pca*-Pol.

**Properties of *Pca*-Pol** The optimal conditions for *Pca*-Pol were analyzed in the presence of activated calf thymus DNA as a template. The optimal pH for enzyme activity was found to be 8.5 in 25 mM Tris–Cl buffer (Fig. 2A). Effect of temperature on activity of *Pca*-Pol was analyzed in a range of 20–100°C. Highest enzyme activity was observed at 75°C (Fig. 2B). This observation does not truly reflect the optimal temperature for the enzyme activity because of decreased stability of substrate DNA at higher temperatures. On the other hand, the polymerase was found to be stable at temperatures higher than 75°C.

When we examined the effect of $MgCl_2$ concentration on *Pca*-Pol activity, no DNA polymerase activity was observed in the absence of $MgCl_2$. The enzyme showed maximum activity at a $MgCl_2$ concentration of 4 mM (Fig. 2C). Presence of monovalent cations such as ammonium and potassium had an inhibitory effect on the enzyme activity of *Pca*-Pol. The enzyme was most active in the absence of these cations (Fig. 2D).

The effect of Triton X-100, Tween 20, BSA and ethylenediaminetetraacetic acid (EDTA) was determined on the activity of *Pca*-Pol. Enzyme assays were conducted in the presence of 4 mM $MgCl_2$. Enzyme activity was slightly enhanced by the presence of 0.1% Tween 20 and 0.2 mg/ml BSA, and completely inhibited in the presence of 3 mM EDTA (data not shown).

We also determined the minimum concentration of dNTPs required for the enzyme activity. The 40-mer primer annealed to 80-mer template was incubated with *Pca*-Pol in the presence of various concentrations of dNTPs. Synthesis of 75-mer product was not observed up to a dNTPs concentration of 100 nM. The formation of the product was detected at a dNTPs concentration of 1 μM and above (Fig. S4).

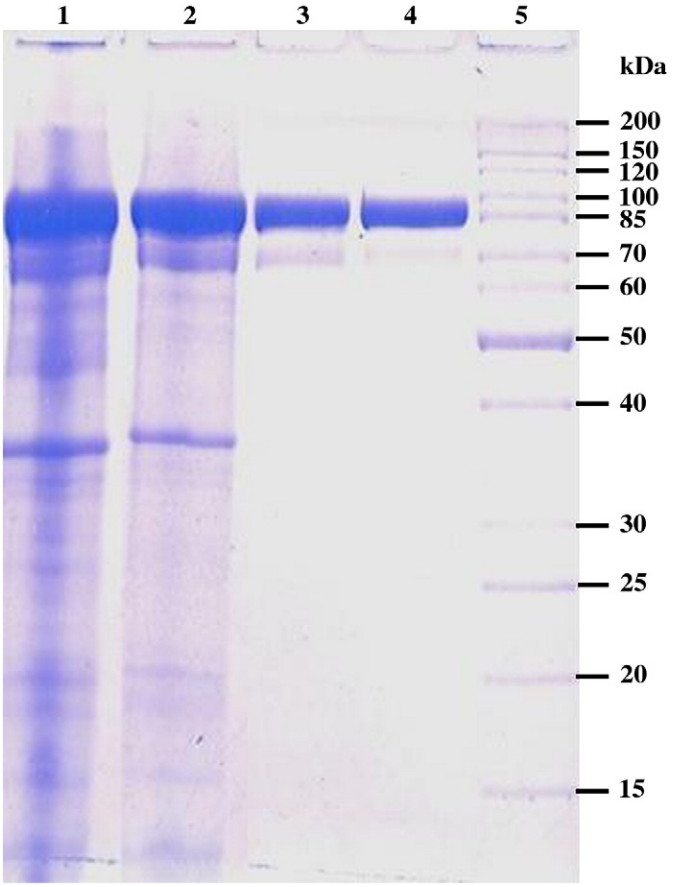



FIG. 1. SDS-PAGE analysis of *Pca*-Pol. Electrophoresis was performed on 12% polyacrylamide gel and stained with Coomassie Brilliant Blue G-250; lane 1, sonicated extract of host cells carrying pol-pET21a; lane 2, supernatant after heating step; lane 3, *Pca*-Pol after ResourceQ column; lane 4, *Pca*-Pol after ResourcePHE column; lane 5, molecular mass marker.

Thermostability of *Pca*-Pol was analyzed by measuring the decrease in polymerase activity after incubating the enzyme at either 95°C or 100°C for various intervals of time. Half-life of the enzyme was found to be 4.5 h and 30 min at 95°C and 100°C, respectively (Fig. 3).

**Effect of inhibitors** Effect of aphidicolin and dideoxythymidine triphosphate (ddTTP) on DNA polymerase activity was also examined. No significant effect was observed at lower concentration of these inhibitors. However, at higher concentrations the enzyme activity was significantly inhibited. Approximately 50% of the enzyme activity was detected in the presence of 900 μM aphidicolin (Fig. 4A).

To examine the effect of ddTTP on the activity of *Pca*-Pol, various concentration ratios of ddTTP to TTP [methyl-$^3$H] were used. Slight inhibition was observed at a concentration ratio of 2. Almost 50% of the enzyme activity could be detected when the ratio was increased to

**TABLE 1.** Production and purification of recombinant *Pca*-Pol.

| Purification step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Fold purification | Recovery (%) |
|---|---|---|---|---|---|
| Crude extract | 368.23 | 24990 | 67.86 | 1 | 100 |
| Heat denaturation | 212.1 | 19140 | 90.24 | 1.33 | 76.59 |
| Anion exchange chromatography | 38.22 | 15060 | 394.03 | 5.81 | 60.26 |
| Hydrophobic interaction chromatography | 6.46 | 7332 | 1133.58 | 16.7 | 29.34 |

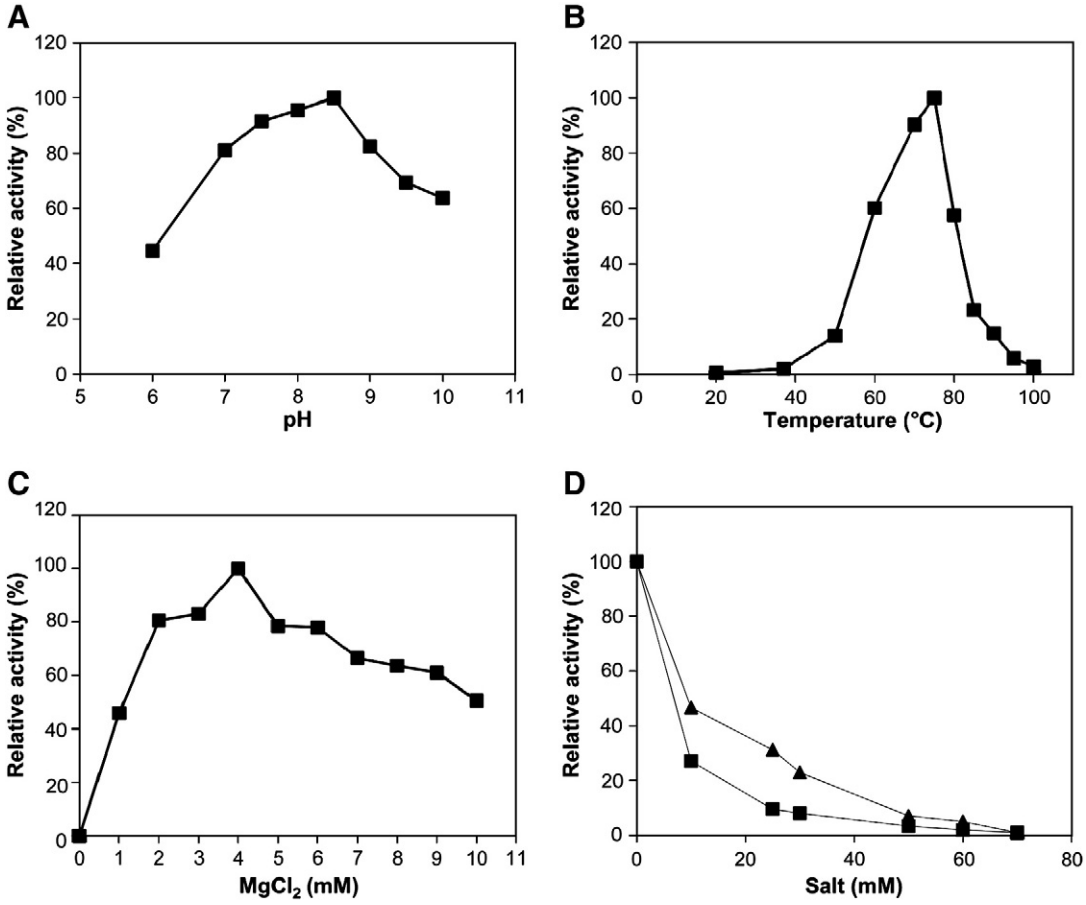



FIG. 2. Characterization of *Pca*-Pol. (A) Effect of pH on *Pca*-Pol activity. Activity was assayed in 25 mM Tris–Cl of various pH values. Buffer pH was measured at 25°C. (B) Effect of temperature on *Pca*-Pol activity. Assay was conducted at various temperatures in 25 mM Tris–Cl pH 8.5. (C) Effect of $MgCl_2$ on *Pca*-Pol activity. Enzyme activity was assayed in 25 mM Tris–Cl pH 8.5 at 75°C. (D) Effect of $(NH_4)_2SO_4$ (squares) and KCl (triangles) on *Pca*-Pol activity. Activity was assayed in 25 mM Tris–Cl pH 8.5 containing 4 mM $MgCl_2$ at 75°C.

10 (Fig. 4B). Family B DNA polymerases show reasonable resistance to dideoxynucleoside triphosphates (18) which was also evident in the case of *Pca*-Pol.

**Exonuclease activity** The fidelity of DNA polymerases is manifested by the presence of 3′→5′ exonuclease activity (20). Almost all archaeal family B DNA polymerases possess 3′→5′ exonuclease activity which is responsible for their improved fidelity (21). We investigated the 3′→5′ exonuclease activity of *Pca*-Pol by incubation of 3′ $^3$H labeled linear DNA with the enzyme. The 3′→5′ exonuclease activity was detected by the decrease in radioactivity of the labeled substrate DNA after incubation with *Pca*-Pol. This is in agreement with the deduced amino acid sequence of the enzyme in which the 3′→5′ exonuclease motifs were identified (Fig. S1).

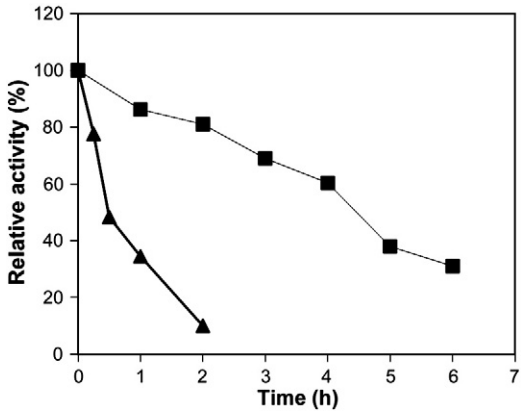



FIG. 3. Thermostability of *Pca*-Pol. The enzyme was incubated at 95°C (squares) and 100°C (triangles) in the absence of dNTPs, BSA and substrate DNA. After indicated time, an aliquot was removed and enzyme activity was monitored by assay procedure described in Materials and methods.

*Pca*-Pol was able to release 65% of radioactivity from 3′-end of the labeled substrate in an hour (Fig. 5A) which is comparable to that released by *Pfu* DNA polymerase in the same time (data not shown). Removal of almost half of the radiolabel was observed in the presence of dNTPs as compared to that in their absence (Fig 5A). Like the DNA polymerase activity, the 3′→5′ exonuclease activity of *Pca*-Pol was found to be inhibited by the presence of ammonium sulfate (Fig. 5B). Inhibition observed was proportional to the concentration of the salt and there was no detectable enzyme activity when 70 mM ammonium sulfate was present in the assay mixture.

We further analyzed 3′→5′ exonuclease activity by degradation of 5′-biotinylated 40-mer oligonucleotide. *Pca*-Pol demonstrated 3′→5′ exonuclease activity on both single and double stranded DNA molecules (Fig. 5C).

**PCR application of *Pca*-Pol** Thermostable DNA polymerases are important for DNA amplification and DNA sequence determination (22). DNA polymerases with associated 3′→5′ exonuclease activity have been shown to amplify DNA with high fidelity (13,21). The *Pca*-Pol possessing 3′→5′ exonuclease activity was evaluated for its use in PCR. DNA amplification of up to 7.5 kb was observed when

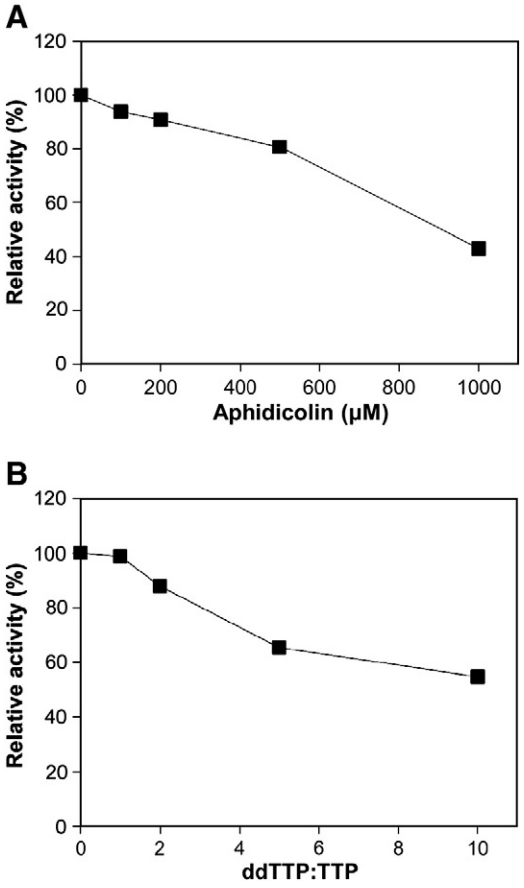
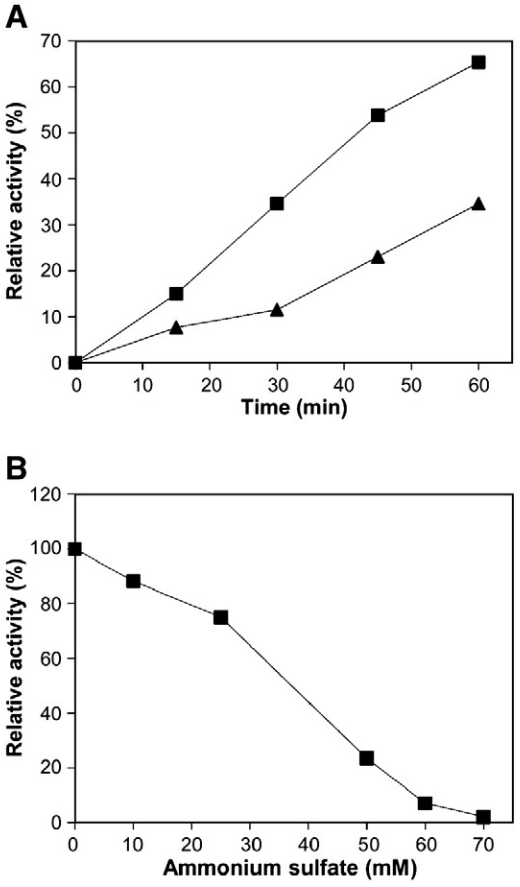



FIG. 4. Effect of inhibitors on *Pca*-Pol activity. Effect presence of (A) aphidicolin and (B) ddTTP. Assay was conducted as described in Materials and methods.



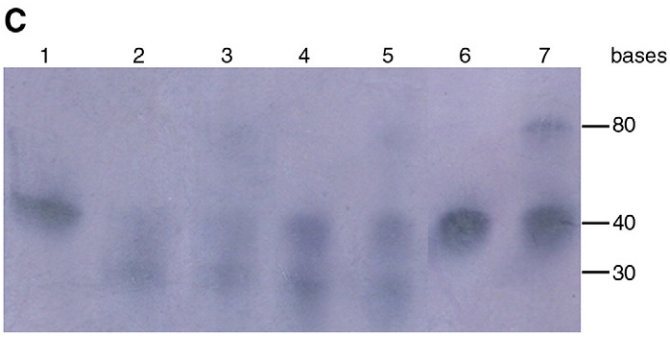




FIG. 5. 3′→5′ exonuclease activity of *Pca*-Pol. (A) Exonuclease activity in the absence (squares) and presence of dNTPs (triangles). (B) Effect of ammonium sulfate on exonuclease activity of *Pca*-Pol. (C) Single and double strand-dependent 3′→5′ exonuclease activity of *Pca*-Pol; lane 1, primer only; lane 2, single stranded; and lane 3, double stranded DNA incubated with *Pca*-Pol; lane 4, single stranded; and lane 5, double stranded DNA incubated with Pfu DNA polymerase; lane 6, single stranded; and lane 7, double stranded DNA incubated with *Taq* DNA polymerase. Length of DNA (bases) is indicated on the right side.

PCR was performed with *Pca*-Pol (Fig. 6). No other DNA polymerase from genus *Pyrobaculum* has been reported to amplify DNA fragments larger then 1.5 kb (18,23). Hence, *Pca*-Pol was able to amplify larger DNA fragments in PCR.

## DISCUSSION

In this study we describe gene cloning, expression and purification of a thermostable DNA polymerase from *P. calidifontis*. When we compared the properties of the enzyme with other DNA polymerases characterized from the genus *Pyrobaculum* (18,23) we found that *Pca*-Pol had a slight basic pH optima in Tris–Cl buffer and it was able to amplify larger DNA fragments. The enzyme required magnesium for its activity and was inhibited by the presence of ammonium and potassium ions. Some other crenarchaeal DNA polymerases have also been reported to be inhibited by monovalent cations (4,18,23). It was also observed that the extent of inhibition by ammonium is greater as compared to that by potassium ions. The PCR amplification by *Pca*-Pol decreased in the presence of ammonium or potassium ions (data not shown). On the other hand, commercially available DNA polymerases like *Pfu*, *Taq* and Vent DNA polymerases require monovalent cations in PCR buffer. The *Pca*-Pol was highly thermostable with a half-life of 4.5 h at 95°C (and 0.5 h at 100°C) which is greater than that of *Taq* DNA polymerase (24) but less than that of *Thermococcus litoralis* DNA polymerase (Vent polymerase) (25). The concentration of dNTPs required for detectable primer elongation was investigated and found to be over 100 nM. Similar finding has been reported for *Thermoanaerobacter yonseiensis* DNA polymerase (26). The *Pca*-Pol was found to possess 3′→5′ exonuclease activity comparable to that of *Pfu* DNA polymerase. Whereas, 3′→5′ exonuclease activity was not detected in case of *Taq* DNA polymerase (data not shown). The 3′→5′ exonuclease activity of some DNA polymerases has been shown to decrease significantly in the presence of dNTPs (4,13,23). But the *Pca*-Pol was able to remove almost half of the radiolabel from 3′-end labeled DNA even in the presence of 1 μM dNTPs (concentration required for detectable primer elongation). Indeed, in case of *P. islandicum* DNA polymerase, significant degradation of double stranded DNA by 3′→5′ exonuclease activity was observed even in the presence of 0.1 μM dNTPs which was reduced at a concentration of 1 μM (18). Similarly, *Thermococcus guaymasensis* DNA polymerase

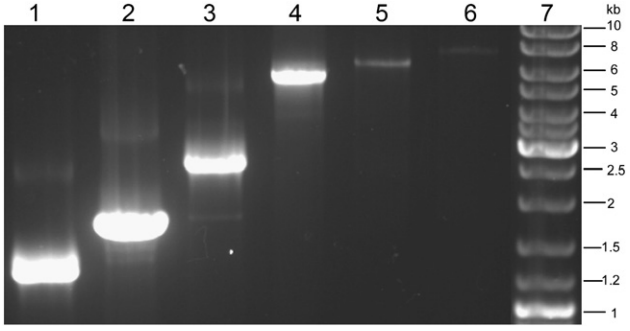



FIG. 6. PCR amplification of DNA fragments of various lengths by *Pca*-pol. Amplification was performed in buffer containing 10 mM Tris–Cl pH 8.5, 4 mM $MgCl_2$, 200 μM each dNTP and 0.2 mg/ml BSA; lane 1, 1.3 kb amplification; lane 2, 1.7 kb amplification; lane 3, 2.6 kb amplification; lane 4, 5.4 kb amplification; lane 5, 6.5 kb amplification; lane 6, 7.5 kb amplification; lane 7, molecular mass marker.

was reported to show 3′→5′ exonuclease activity in the presence of dNTPs (21) and Kong et al. showed that *T. litoralis* DNA polymerase demonstrated half of the maximal 3′→5′ exonuclease activity in the presence of 1 μM dNTPs (25). The 3′→5′ exonuclease activity enables the enzyme to replicate DNA accurately. Inactivation of 3′→5′ exonuclease activity results in an increased error rate (27,28). Together, these data suggest that the *Pca*-Pol could be able to replicate DNA with a lesser error rate.

The *Pca*-Pol was applied to PCR and amplification of DNA fragments of up to 7.5 kb was observed. Although several crenarchaeal DNA polymerases have been studied (4,13,18,23,29,30) but not all have been used in PCR. Amplification of 0.5 and up to 1.5 kb has been demonstrated in case of *Pyrobaculum arsenaticum* (23) and *P. islandicum* (18) DNA polymerases, respectively. Likewise, *Sulfophobococcus zilligii* DNA polymerase was shown to amplify 0.5 kb DNA fragment (4) and *Staphylothermus marinus* DNA polymerase was able to amplify DNA fragments of up to 6 kb (13). Our results show that *Pca*-Pol could amplify greater target lengths in PCR as compared to several other crenarchaeal DNA polymerases. Also the enzyme was able to incorporate biotin-11-UTP and digoxigenin-11-UTP in DNA—both nucleotides used for non-radioactive DNA labeling. Therefore, *Pca*-Pol could be a suitable enzyme for amplification of larger DNA fragments with high fidelity.

Supplementary materials related to this article can be found online at doi:10.1016/j.jbiosc.2011.03.018.

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