Cloning, Expression, and Characterization of a Family B-Type DNA Polymerase from the Hyperthermophilic Crenarchaeon *Pyrobaculum arsenaticum* and Its Application to PCR

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Abstract The gene encoding *Pyrobaculum arsenaticum* DNA polymerase (*Par* DNA polymerase) was cloned and sequenced. The gene consists of 2,361 bp coding for a protein with 786 amino acid residues. The deduced amino acid sequence of *Par* DNA polymerase showed a high similarity to archaenal family B-type DNA polymerases (Group I), and contained all of the motifs conserved in the family B-type DNA polymerases for 3'→5' exonuclease and polymerase activities. The *Par* DNA polymerase gene was expressed under the control of the T7lac promoter on the expression vector pET-22b (+) in *Escherichia coli* BL21-CodonPlus(DE3)-RP. The expressed enzyme was purified by heat treatment, and Cibacron blue 3GA and HiTrap™ Heparin HP column chromatographies. The optimum pH of the purified enzyme was 7.5. The enzyme activity was activated by divalent cations, and was inhibited by EDTA and monovalent cations. The half-life of the enzyme at 95°C was 6 h. *Par* DNA polymerase possessed associated 3'→5' proofreading exonuclease activity, which is consistent with its deduced amino acid sequence. PCR experiment with *Par* DNA polymerase showed an amplified product, indicating that this enzyme might be useful in DNA amplification and PCR-based applications.

Keywords: Archaea, DNA polymerase, exonuclease activity, polymerase chain reaction, *Pyrobaculum arsenaticum*, thermostable enzyme

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Hyperthermophiles are organisms that grow optimally at 80°C or higher temperatures. The thermostability exhibited by hyperthermophiles has been the subject of considerable interest for both academic research and industrial applications. Among the different applications, the most salient application may be the polymerase chain reaction (PCR) using thermostable DNA polymerases [31]. PCR has become a powerful method for the identification and amplification of genes, for direct sequencing, and for clinical diagnosis [14]. A thermostable DNA polymerase is much more suitable for the thermocycle during PCR [14, 31]. Accordingly, a thermostable DNA polymerase has become an indispensable ingredient of PCR technology.

DNA polymerases play leading roles in cellular DNA replication and repair. Since the discovery and characterization of DNA polymerase I from *E. coli* by Kornberg and colleagues in the 1950s [22], a large number of DNA polymerases have been isolated and identified from prokaryotic and eukaryotic sources. These DNA polymerases have been classified into five major groups based on the alignment of amino acid sequences [5, 8]; families A, B, C, D, and X are represented by *E. coli* DNA polymerases I, II, III α subunit, *Pyrococcus furiosus* DNA polymerase II, and others such as eukaryotic DNA polymerase β, respectively. The majority of archaenal DNA polymerases have been identified as members of family B, along with the eukaryotic replicative DNA polymerases [8]. Archaenal family B-type DNA polymerases could be further divided into at least two subfamilies of Groups I and II; Group I includes all euryarchaeote DNA polymerases and several crenarchaeote DNA polymerases, and Group II encompasses the remaining crenarchaeote DNA polymerases.

Thermostable DNA polymerase was first isolated from the thermophilic bacterium *Thermus aquaticus* YT-1, and its properties have been reported [9]; Taq DNA polymerase does not possess 3'→5' exonuclease activity. Thermostable DNA polymerases with an integrated 3'→5' exonuclease
activity have been identified from the hyperthermophilic
euryarchaeotes, P. furiosus [26] and Thermococcus litoralis
[28], and the hyperthermophilic crenarchaeotes, Pyrodictium
occultum [37], Aeropyrum pernix [7], and Pyrobaculum
islandicum [21]. The increasing number of applications
utilizing PCR has generated an increasing demand for
various thermostable DNA polymerases.

The domain Archaea has been recognized as a third
major branch of living organisms, with the other two
branches being the Bacteria and the Eukarya [39]. Although
the Archaea is referred to as prokaryote together with
the Bacteria, some rooted-phylogenetic trees of life have
implied that the Archaea and Eukarya are connected by a
common ancestor [6]. From a phylogenetic perspective
based on rRNA sequences, the Archaea has been classified
into four phyla [3, 18]: the Crenarchaeota, Euryarchaeota,
Korarchaeota, and Nanoarchaeota, a phylum which has
only recently been identified.

The hyperthermophilic crenarchaeon Pyrobaculum
arsenatum was isolated from a hot spring at Pisciairelli
Solfatara, Naples, Italy [19]. P. arsenatum is a rod-
shaped, strictly anaerobic, facultative organotrophic strain.
This organism can grow chemoautotrophically using
carbon dioxide as a carbon source, hydrogen as an electron
donor, and arsenate, thiosulfate, or elemental sulfur as an
electron acceptor. No information is currently available
with regard to the nucleotide sequence of any protein-
encoding gene and the biochemical characteristics of any
protein from P. arsenatum, perhaps owing to the difficulty
of cultivating this hyperthermophilic, strictly anaerobic
strain.

In this study, we describe the cloning and expression of
the gene encoding P. arsenatum family B-type DNA
polymerase (Par DNA polymerase). We also report the
purification and properties of the enzyme. Finally, we
present the results on the PCR experiment with Par DNA
polymerase.

MATERIALS AND METHODS

Strains and Culture Conditions

P. arsenatum (DSM 13514) was taken from the German
Collection of Microorganisms and Cell Cultures (Deutsche
Sammlung von Mikroorganismen und Zellkulturen GmbH,
DSMZ). The P. arsenatum cells were grown in DSMZ
Medium 390 as follows. Medium, containing (per liter)
1.3 g of (NH₄)₂SO₄, 0.28 g of KH₂PO₄, 0.25 g of MgSO₄·
7H₂O, 0.07 g of CaCl₂·2H₂O, 0.02 g of FeCl₃·6H₂O, 1.8 mg
of MnCl₂·4H₂O, 4.5 mg of Na₂B₄O₇·10H₂O, 0.22 mg of
ZnSO₄·7H₂O, 0.05 mg of CuCl₂·2H₂O, 0.03 mg of Na₂MoO₄·
2H₂O, 0.03 mg of VOSO₄·2H₂O, 0.01 mg of CoSO₄, 2 g of
Na₂S·9H₂O, 5H₂O, and 1 mg of resazurin, was adjusted to pH
6.0 with 8 N NaOH, and was gassed with N₂ for 30 min.
The medium was supplemented with (per liter) 0.5 g of
bacto-peptone, 0.2 g of bacto-yeast extract, and 0.5 g of
Na₂S·9H₂O, and was readjusted to pH 6.0 with 10 N
H₂SO₄. Following the dispensation under N₂ gas into
100 ml in a 125-ml serum bottle, the medium was stored at
95°C for 3 days. The cells were then inoculated via syringe
and grown anaerobically at 95°C for 5 days without
shaking.

E. coli MV1184 was used for plasmid propagation and
nucleotide sequencing. E. coli BL21-CodonPlus(DE3)-RP
(Stratagene, La Jolla, CA, U.S.A.), which harbors the T
RNA polymerase gene under the control of a chromosomal
lacUV5 gene [35], was used for gene expression. The E.
coli cells were grown in Luria-Bertani medium with
appropriate antibiotics at 37°C with vigorous shaking.

Genomic DNA Isolation and Hybridization

The genomic DNA of P. arsenatum was isolated by the
method of Marmur [27] with slight modifications. For the
amplification of a Par DNA polymerase gene fragment,
two degenerate primers were designed based on the conserved
Pol I and Pol II motifs of archaeal family B-type DNA
polymerases [21]: the forward primer PolF, 5'-GAATCGAG
(C/T)TTGACTGG(C/A)T(C/G)N(A/T)(C/G)NATGTA
(C/T)CC-3', and the reverse primer PolR, 5'-AANA(A/G)
N(C/G)(A/T)(A/G/TCNGT(A/G)TCNCC(A/G)TA-3'.
DNA amplification was carried out using 2.5 U of Pfu
DNA polymerase in a 50-μl reaction mixture, consisting of
10 ng of P. arsenatum genomic DNA, 50 pmol of each
primer, 200 μM dNTPs, and PCR reaction buffer. After a
single 3 min denaturation step at 95°C, PCR (30 cycles)
did not require denaturation at 94°C for 45 sec, annealing at
45°C for 60 sec, and extension at 72°C for 1 sec. A single
3 min extension at 72°C was performed before terminating
the reaction. A predicted 440-bp PCR product was then
purified from 2% low melting agarose gel and labeled with
[α-³²P]dCTP by random priming, to make the probe used for
the DNA-DNA hybridization, which could detect the
complete Par DNA polymerase gene. DNA hybridization
was performed by the agarose gel membrane hybridization
method [34]. P. arsenatum genomic DNAs digested with
three restriction enzymes (EcoRI, HindIII, or SacI) were
fractionated on 0.7% agarose gel, and the probe was
hybridized at 53°C to the fractionated genomic DNA on
the agarose gel membrane. Colony hybridization was
conducted according to the standard procedures [16], using
the aforementioned ³²P-labeled probe. DNA inserts of
positive clones were characterized following plasmid DNA
extraction.

DNA Sequencing and Sequence Analysis

The nucleotide sequences of the purified PCR product and
hybridized DNA were determined using an Applied
Biosystems 3730 DNA sequencer (Foster City, CA, U.S.A.).
The obtained sequences were compared with known proteins in the database using the sequence comparison program, BLAST. Nucleotide and deduced amino acid sequence analyses were performed using the DNASIS (Hitachi Software Engineering, Japan) and PGENE (IntelliGenetics, U.S.A.) softwares. The MultAlin program [12] was used for multiple sequence alignment between functionally related proteins.

Construction of Plasmid Expressing Par DNA Polymerase
Most of the methods used for plasmid construction were predicated on those described by Sambrook et al. [32]. For the expression of Par DNA polymerase, the DNA polymerase gene was amplified by direct PCR of the P. arseniticum genomic DNA. On the basis of the nucleotide sequence of the Par DNA polymerase gene, two primers were synthesized: the 5′ (N-terminal) primer, PPoolNN, 5′-CTGTTGTCATATAGGAGATCCGGTGT-3′, a unique Ndel site (underlined) added, which has the translation initiation codon, ATG; and the 3′ (C-terminal) primer, PPoolCH, 5′-CGTAAAGCCTTCTAATCGAGTCAGACA-3′, which matches the C-terminal sequence including the stop codon, a unique HindIII site (underlined) added. The amplified fragment containing the Par DNA polymerase sequence was digested with Ndel and HindIII, purified from 0.8% low melting agarose gel, and ligated into the expression vector pET-22b(+) (Novagen, Madison, WI, U.S.A.), which had been digested with the same enzymes. E. coli BL21-CodonPlus(DE3)-RP was transformed with the ligate by electroporation. Clones with the correct construct were selected by restriction enzyme analysis of plasmid minipreps.

Purification of the Expressed Par DNA Polymerase in E. coli
A 10 ml of overnight culture of E. coli BL21-CodonPlus(DE3)-RP harboring the relevant expression plasmid, which was grown in Luria-Bertani broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol, was transferred to 1 l of the same medium. This culture was grown at 37°C until an A_600 of 0.6 was reached. Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was grown for another 6 h [17, 24]. The cells were harvested by centrifugation and resuspended in buffer A (10 mM Tris-HCl, pH 7.5, and 1 mM MgCl_2) containing 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The resuspended cells were disrupted by sonication and centrifuged at 35,000 ×g, 4°C for 20 min to remove E. coli cell walls and insoluble debris. DNaseI was added to the sonicated extract at a final concentration of 0.5 μg/ml, and the mixture was incubated at 37°C for 30 min. The majority of heat-labile E. coli proteins was precipitated by heat treatment at 85°C for 30 min. After removal of the denatured proteins by centrifugation at 35,000 ×g, 4°C for 20 min, the supernatant was dialyzed against buffer A, and was then applied onto a Cibacron blue 3GA column pre-equilibrated with buffer A. The column was washed with 10 column volumes of buffer A, and developed with a linear gradient of 0–1000 mM KCl prepared in buffer A. Major fractions containing the desired protein were pooled and dialyzed against buffer A. Par DNA polymerase was further purified by a HiTrap™ Heparin HP column (Amersham Biosciences, Uppsala, Sweden). After loading of the sample, the column was washed with 10 column volumes of buffer A, and protein was eluted with a linear gradient of 0–1500 mM KCl prepared in buffer A. The purified enzyme was desalted by dialysis against buffer A.

Protein concentration was determined by the method of Lowry et al. [25] with bovine serum albumin (BSA) as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, as previously described by Laemmli [23], with 8% polyacrylamide gel.

DNA Polymerase Activity Assay
The DNA polymerase activity of the purified enzyme was measured as described by Choi and Kwon [11] with slight modifications. The basic reaction mixture (50 μl) contained 25 mM Mops-NaOH (pH 7.5), 2 mM MgCl_2, 1 mM 2-mercaptoethanol, 100 μM each dATP, dCTP, and dGTP, 10 μM dTTP, 0.5 μCi of [methyl-3H]thymidine 5′-triphosphate, 1.25 μg of activated calf thymus DNA, and enzyme solution. This mixture was incubated at 75°C for 10 min. The reaction was stopped on ice, and an aliquot was spotted onto a DE81 filter paper disc (23 mm, Whatman, Maidson, U.K.). The disc was dried on a heat block, and washed in 0.5 M sodium phosphate (pH 7.0) buffer for 10 min and in 70% ethanol for 5 min, and then dried. The incorporated radioactivity of the dried filter paper disc was counted using a Beckman LS6500 scintillation counter (Fullerton, CA, U.S.A.). One unit of Par DNA polymerase is defined as the amount of polymerase that incorporates 10 pmole of [3H]TTP into an acid-insoluble product at 75°C in 10 min.

Exonuclease Activity Assay
To prepare the 3′ end-labeled DNA substrate, pBluescript SK(-) DNA linearized by NotI was filled in by Klenow fragment in the presence of [α-32P]dTTP. To prepare the 5′ end-labeled DNA substrate, pBluescript SK(-) DNA linearized by Smal was phosphorylated by T4 polynucleotide kinase in the presence of [γ-32P]ATP. After labeling, each DNA substrate was purified by gel filtration on a Sephadex G-25 column, followed by ethanol precipitation. For exonuclease activity assay, end-labeled DNA substrate was placed in 25 mM Tricine-NaOH (pH 7.5), 5 mM MgCl_2, 0.01% BSA, and enzyme solution. The reaction mixture (50 μl) was incubated at 75°C in the presence or absence of dNTPs. The reaction was stopped on ice and precipitated by the addition of 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 [11].

**PCR with the Purified Par DNA Polymerase**

Oligonucleotide primers that annealed to λ-phage DNA [33] were designed to give 500-bp amplified DNA fragment by PCR: the forward primer 5'-CGCGACAGCTAGAA-CAGAC-3' and the reverse primer 5'-GCGGCCGACGCATAAACGAA-3'. PCR was carried out with 1 unit of the purified Par DNA polymerase in a 50-µl reaction mixture containing 50 ng of λ-phage DNA, 5 pmol of each primer, 200 µM dNTPs, and PCR reaction buffer. After a single 5 min denaturation step at 95°C, 30 cycles with a temperature profile of 30 sec at 94°C, 30 sec at 60°C, and 60 sec at 72°C were performed, followed by a final 5 min extension at 72°C.

**RESULTS AND DISCUSSION**

Cloning, Sequencing, and Sequence Analysis of the Par DNA Polymerase Gene

A DNA fragment of approximately 440 bp in length was amplified from P. arsenicophilum genomic DNA by PCR with two degenerate primers based on the conserved Pol I and Pol II motifs from archaean family B-type DNA polymerases. As expected, the deduced amino acid sequence from the nucleotide sequence of the PCR product showed a marked similarity to archaeal family B-type DNA polymerases. To clone the Par DNA polymerase gene, the PCR product was used as a hybridization probe. Agarose gel membrane hybridization of the restriction enzyme digests of the P. arsenicophilum genomic DNA revealed an approximately 5.5-kb HindIII fragment, which hybridized with the 32P-labeled probe (data not shown). The region containing the fragment was excised from a gel and ligated into pBluescript SK(-), and then E. coli MV1184 was transformed with the ligate. After colony hybridization, the insert in the plasmid extracted from the positive clone was sequenced using a primer walking method. The Par DNA polymerase gene consists of 2,361 bp coding for a protein with 786 amino acid residues (data not shown). The gene starts with a valine codon, GTG, like the P. islandicum DNA polymerase gene [21]. It was reported by the survey of open reading frames from Methanococcus that about 25% and 5% of archaeal proteins appeared to start at GTG and TTG, respectively, instead of ATG [13]. The nucleotide sequence of the Par DNA polymerase gene was deposited in GenBank under accession number AY914798.

Consensus archaeabacterial promoter sequence was found on the upstream flanking region of the Par DNA polymerase gene. The TATAATA sequence at 26 bases upstream from the open reading frame was homologous to the box A [TTTA(A/T)A] promoter sequence [30]. The G+C content of the Par DNA polymerase gene was calculated to be 57.0%. This value was similar to 58.3 mol% of the G+C content determined in the genomic DNA [19]. The G+C content in the third position of codons was higher, at 73.3%, compared with 60.4% and 37.4% in the first and second positions, respectively, of codons. Most DNA polymerase-encoding genes from (hyper)thermophilic cobacteria and archaeabacteria have shown that G and C were preferentially used in the third position of codons [11, 20, 37].

**Similarity Analysis of the Amino Acid Sequence of Par DNA Polymerase**

The deduced primary structure of Par DNA polymerase was aligned and compared with those of archaeal family B-type DNA polymerases.

![Fig. 1. Amino acid sequence alignment of archaeal family B-type DNA polymerases.](image-url)
B-type DNA polymerases that showed a high similarity (Fig. 1). Amino acid sequence alignment revealed that the Par DNA polymerase contains all of the conserved motifs, including the three 3'→5' exonuclease motifs [4], the six 5'→3' polymerase motifs [5], and the DNA binding motif Y-G(G/A) [36], which are highly conserved among archaeal family B-type DNA polymerases. The presence of highly conserved motifs in Par DNA polymerase suggests that this enzyme possesses 3'→5' exonuclease activity and 5'→3' polymerase activity. The deduced amino acid sequence of Par DNA polymerase is closely related to those of crenarchaeotal family B-type DNA polymerases belonging to Group I. Par DNA polymerase showed 81.0% similarity to Pyrobaculum aerophilum DNA polymerase B3 [15], 52.8% to P. occultum DNA polymerase II [37], and 35.6% to P. furiosus DNA polymerase [38].

**Expression and Purification of Par DNA Polymerase**

The pET system is one of the most powerful systems developed for the cloning and expression of recombinant proteins in E. coli [35]. The pET-22b(+) vector has a very strong and stringent T7lac promoter. The Par DNA polymerase gene was amplified and inserted into the NdeI and HindIII site of pET-22b(+). The resulting expression plasmid was designated pEPAP. The culture of E. coli BL21-CodonPlus(DE3)-RP harboring pEPAP was done in a 1-L fermenter. Par DNA polymerase was expressed as a soluble form in the cytosol. The harvested cells (3.69 g) were initially sonicated and treated with DNaseI. We then utilized the thermophilic property of Par DNA polymerase, and eliminated most E. coli proteins by heating at 85°C for 30 min and centrifuging to remove all of the denatured proteins [1]. Several E. coli proteins still remained soluble after the heating step. The soluble supernatant from the heating step was then chromatographed on a Cibacron blue 3GA column and a HiTrap™ Heparin HP column. The purification of the enzyme is summarized in Table 1. The specific activity of the purified enzyme was determined to be 3,634.6 units/mg, and recovery was approximately 24.3% on the basis of the total activity of the sonicated extract. The purification of the enzyme was monitored by SDS-PAGE (Fig. 2). The SDS-PAGE revealed a single protein band with a molecular mass of 90,000 Da, which was in good agreement with the sum of the molecular mass, 89,525 Da, of Par DNA polymerase calculated from the deduced amino acid sequence.

![Fig. 2. SDS-PAGE analysis of Par DNA polymerase. Electrophoresis was performed on a vertical gel of 8% polyacrylamide. Lane M, low-molecular-mass markers (molecular masses are indicated at the left); lane E, purified enzyme.](image)

**Properties of Par DNA Polymerase**

The dependence of the Par DNA polymerase activity on pH was determined in the pH range of 6.0–10.0. The buffers used were 25 mM Mops-NaOH (pH 6.0–8.0), 25 mM Tricine-NaOH (pH 7.5–9.5), and 25 mM Glycine-NaOH (pH 9.0–10.0). The enzyme activity was highest at pH 7.5 (Fig. 3A). In contrast, other commercial thermostable DNA polymerases used in PCR, such as Taq DNA polymerase, Pfu DNA polymerase, and Vent DNA polymerase, are recommended to perform the reaction at alkaline pH values, generally at the pH range of 8.5–9.0. The dependence of the Par DNA polymerase activity on temperature was determined in the range of 50–90°C. The maximal activity of polymerization by Par DNA polymerase was observed at 75°C on an activated calf thymus DNA template (Fig. 3B). This maximum appears to reflect the denaturation of the template at higher temperatures, because the enzyme was significantly stable at 95°C; therefore, the optimum temperature of the enzyme would actually be above 75°C.

**Table 1.** Purification scheme of Par DNA polymerase expressed in E. coli.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated extract</td>
<td>242.0</td>
<td>120,914</td>
<td>499.6</td>
<td>100</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>26.4</td>
<td>73,737</td>
<td>2,793.1</td>
<td>61.0</td>
</tr>
<tr>
<td>Cibacron blue 3GA</td>
<td>11.7</td>
<td>39,109</td>
<td>3,342.6</td>
<td>32.3</td>
</tr>
<tr>
<td>HiTrap™ heparin</td>
<td>8.1</td>
<td>29,440</td>
<td>3,634.6</td>
<td>24.3</td>
</tr>
</tbody>
</table>
The effects of divalent cations on the Par DNA polymerase activity were examined with various concentrations of MgCl₂ and MnCl₂. The optimal Mg²⁺ concentration was 5 mM, and the optimal Mn²⁺ concentration was 1 mM (Fig. 4A). No activity was detected in the absence of divalent cations, indicating that divalent cations are required for the polymerization reactions to occur. The activation of Par DNA polymerase by Mg²⁺ ion is consistent with the effects of this cation on other DNA polymerases [11]. Most DNA binding enzymes, including DNA-dependent DNA polymerases, tend to prefer the presence of Mg²⁺ ion. Interestingly, the maximal activity of Par DNA polymerase by Mn²⁺ ion was similar to that by Mg²⁺ ion. In contrast, almost all of the DNA polymerases have been shown to have lower activity in the presence of Mn²⁺ than Mg²⁺ [10, 11]. The effect of Mn²⁺ ion on the catalytic activity of Par DNA polymerase will further be studied. The influences of monovalent cations on the Par DNA polymerase activity were tested with various concentrations of KCl and (NH₄)₂SO₄. The monovalent cations, potassium ion and ammonium ion, inhibited the enzyme activity (Fig. 4B). The inhibition by monovalent cations was also observed in P. islandicum DNA polymerase [21]; additionally, it was reported that the enzyme activity of P. occultum DNA polymerase II decreased sharply in proportion to the
increase of KCl concentration [37]. These results suggest that archaeal family B-type DNA polymerases belonging to Group I can be distinguished from other groups by virtue of the inhibitory effects of monovalent cations.

The effects of EDTA (0–5 mM), DTT (0–10 mM), BSA (0–0.1%), and Triton X-100 (0–1%) on the Par DNA polymerase activity were examined by assaying in the presence of various concentrations of these substances (data not shown). As expected, the enzyme activity was completely inhibited by 1.5 mM EDTA. DTT had no significant influence on the enzyme activity. Par DNA polymerase was stabilized by BSA and Triton X-100 with an approximately 15% increase in the enzyme activity.

The thermostability of Par DNA polymerase was tested by measuring the decrease in activity after preincubation at two different temperatures: 75°C and 95°C. Its thermostability was substantially high, as was expected from a thermostable protein. The enzyme was fairly stable at 75°C, and was comparatively stable at 95°C. The half-life of the enzyme at 95°C was found to be 6 h in the presence of 0.01% BSA (Fig. 5).

**Exonuclease Activities of Par DNA Polymerase**

The incubation of DNA polymerase with linear DNA fragments in the absence of dNTPs resulted in the degradation of the fragments, indicating the existence of an exonuclease activity in the preparation. This activity was verified by assaying the 32P-labeled product released from an end-labeled DNA substrate. When the substrate was labeled on its 3' end, about 83% of the 32P was released in 1 h; however, when the substrate was labeled on its 5' end, no 32P was released (Fig. 6). This result demonstrates that Par DNA polymerase possesses a high 3'→5' exonuclease activity, but no 5'→3' exonuclease activity, which is consistent with its deduced amino acid sequence. Almost all of the archaeal family B-type DNA polymerases are known to possess associated 3'→5' exonuclease activity [21]. Organisms living at very high temperatures may be under unusually strong evolutionary pressure to maintain those genes that specify phenotypic characteristics critical to life there. The existence of a DNA polymerase with a high 3'→5' proofreading exonuclease activity is, therefore, necessary and crucial for the survival of hyperthermophiles.

**PCR with Par DNA Polymerase**

Thermostable DNA polymerases are not necessary to replenish the enzyme after each PCR cycle, as with *E. coli* DNA polymerase I. Unlike *Taq* DNA polymerase prevalently used in PCR, DNA polymerases with the associated 3'→5' exonuclease activity offer the possibility to amplify DNA fragments with high fidelity, although such DNA polymerases are not helpful for rapid and long amplification of DNA. However, this weakness of DNA polymerases with the associated 3'→5' exonuclease activity has been overcome by the optimization of reaction buffer, the construction of mutant with decreased 3'→5' exonuclease
activity, and the use of a mixture of exonuclease-free DNA polymerase and highly proofreading DNA polymerase [2, 29].

PCR experiment was performed with the thermostable Par DNA polymerase having 3'→5' exonuclease activity in the buffer containing 25 mM Tricine-NaOH (pH 7.5), 5 mM MgCl₂, and 0.01% BSA as a stabilizer, based on the results of the above enzyme assays. Par DNA polymerase readily produced the 500-bp DNA fragment (Fig. 7). This result indicates that the enzyme might be useful in DNA amplification and PCR-based applications. Almost all of the archaean family B-type DNA polymerases used in PCR are derived from euryarchaeotes; the crenarchaeotal family B-type DNA polymerases used in PCR are few in number, the most notable example of such being the P. islandicum DNA polymerase [21]. The result from PCR with Par DNA polymerase is, therefore, one of the rare examples, showing successful application of a crenarchaeotal DNA polymerase in PCR.

We have cloned, expressed, and characterized a family B-type DNA polymerase from the hyperthermophilic crenarchaeon P. arsenaticum. This study is the first, to our knowledge, to present information regarding the cloning and expression of a gene from P. arsenaticum, and is also the first to report data regarding the purification and biochemical characteristics of a protein within this strain. We are currently conducting experiments to establish the optimal conditions for long and accurate PCR using Par DNA polymerase, based on the results described in this paper.

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REFERENCES


