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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 archaeal 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 T7*lac* 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.

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

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 thermocycles 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 archaeal DNA polymerases have been identified as members of family B, along with the eukaryotic replicative DNA polymerases [8]. Archaeal 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

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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 arsenaticum* was isolated from a hot spring at Pisciarelli Solfatara, Naples, Italy [19]. *P. arsenaticum* is a rod-shaped, strictly anaerobic, facultative organotrophic strain. This organism can grow chemolithoautotrophically 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. arsenaticum*, 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. arsenaticum* 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. arsenaticum (DSM 13514) was taken from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ). The *P. arsenaticum* cells were grown in DSMZ Medium 390 as follows. Medium, containing (per liter) 1.3 g of $(NH_4)_2SO_4$, 0.28 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.07 g of $CaCl_2 \cdot 2H_2O$, 0.02 g of $FeCl_3 \cdot 6H_2O$, 1.8 mg of $MnCl_2 \cdot 4H_2O$, 4.5 mg of $Na_2B_4O_7 \cdot 10H_2O$, 0.22 mg of $ZnSO_4 \cdot 7H_2O$, 0.05 mg of $CuCl_2 \cdot 2H_2O$, 0.03 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.03 mg of $VOSO_4 \cdot 2H_2O$, 0.01 mg of $CoSO_4$, 2 g of $Na_2S_2O_3 \cdot 5H_2O$, and 1 mg of resazurin, was adjusted to pH 6.0 with 8 N NaOH, and was gassed with N_2 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 $Na_2S \cdot 9H_2O$, and was readjusted to pH 6.0 with 10 N H_2SO_4. Following the dispensation under N_2 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 T7 RNA polymerase gene under the control of a chromosomal *lac*UV5 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. arsenaticum* 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'-(C/T)TNGA(C/T)TT(C/T)(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. arsenaticum* genomic DNA, 50 pmol of each primer, 200 μM dNTPs, and PCR reaction buffer. After a single 5 min denaturation step at 95°C, PCR (30 cycles) was done by denaturation at 94°C for 45 sec, annealing at 45°C for 60 sec, and extension at 72°C for 120 sec. A final 5 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-^{32}P]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. arsenaticum* genomic DNAs digested with three restriction enzymes (*Eco*RI, *Hin*dIII, or *Sac*I) 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 ^{32}P-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 PCGENE (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. arsenaticum* genomic DNA. On the basis of the nucleotide sequence of the *Par* DNA polymerase gene, two primers were synthesized: the 5' (N-terminal) primer, PPolNN, 5'-CTGTGGTCATATGGAGATCCGGGTTT-3', a unique *Nde*I site (underlined) added, which has the translation initiation codon, ATG; and the 3' (C-terminal) primer, PPolCH, 5'-CGTAAAGCTTTCACTCAAAGAAGTCAAGCAA-3', which matches the C-terminal sequence including the stop codon, a unique *Hin*dIII site (underlined) added. The amplified fragment containing the *Par* DNA polymerase gene was digested with *Nde*I and *Hin*dIII, 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 preequilibrated with buffer A. The column was washed with 10 column volumes of buffer A, and developed with a linear gradient of 0–1,000 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 0–1,000 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*-^{3}H]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 [^{3}H]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 *Not*I was filled by Klenow fragment in the presence of [α-^{32}P]dCTP. To prepare the 5' end-labeled DNA substrate, pBluescript SK(-) DNA linearized by *Sma*I was phosphorylated by T4 polynucleotide kinase in the presence of [γ-^{32}P]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'-CGCCACGACGATGAA-CAGAC-3' and the reverse primer 5'-GCCGCCAGCGC-ATAAACGAA-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. arsenaticum* genomic DNA by PCR with two degenerate primers based on the conserved Pol I and Pol II motifs from archaeal 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. arsenaticum* genomic DNA revealed an approximately 5.5-kb *Hin*dIII fragment, which hybridized with the ^{32}P-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 archaebacterial promoter sequence was found on the upstream flanking region of the *Par* DNA polymerase gene. The TTAATA 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 eubacteria and archaebacteria 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

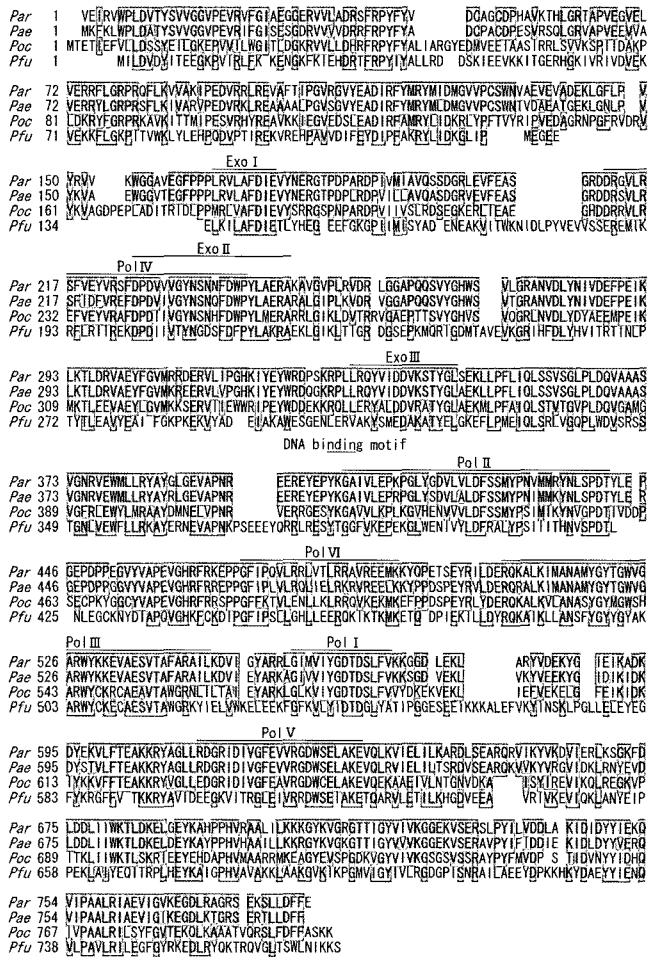

Fig. 1. Amino acid sequence alignment of archaeal family B-type DNA polymerases.

The sequence of *Par* DNA polymerase (*Par*) is shown as compared with those of *P. aerophilum* DNA polymerase B3 (*Pae*) [15], *P. occultum* DNA polymerase II (*Poc*) [37], and *P. furiosus* DNA polymerase (*Pfu*) [38]. Identical amino acids between *Par* DNA polymerase and others are indicated by stippled boxes. The highly conserved motifs among the family B-type DNA polymerases, six 5'→3' polymerase motifs (Pol I through Pol VI), three 3'→5' exonuclease motifs (Exo I, II, and III), and DNA binding motif, are marked.

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 T7*lac* promoter. The *Par* DNA polymerase gene was amplified and inserted into the *Nde*I and *Hin*dIII 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.

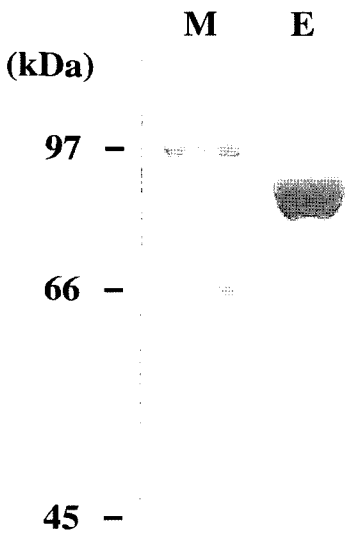

Fig. 2. SDS-PAGE analysis of *Par* DNA polymerase.
Electrophoresis was performed on a vertical gel of 8% polyacrylamide. Lane M, low-molecular-mass markers (molecular masses are indicated at the left); lane E, purified enzyme.

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*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Sonicated extract	242.0	120,914	499.6	100
Heat treatment	26.4	73,737	2,793.1	61.0
Cibacron blue 3GA	11.7	39,109	3,342.6	32.3
HiTrap™ heparin	8.1	29,440	3,634.6	24.3

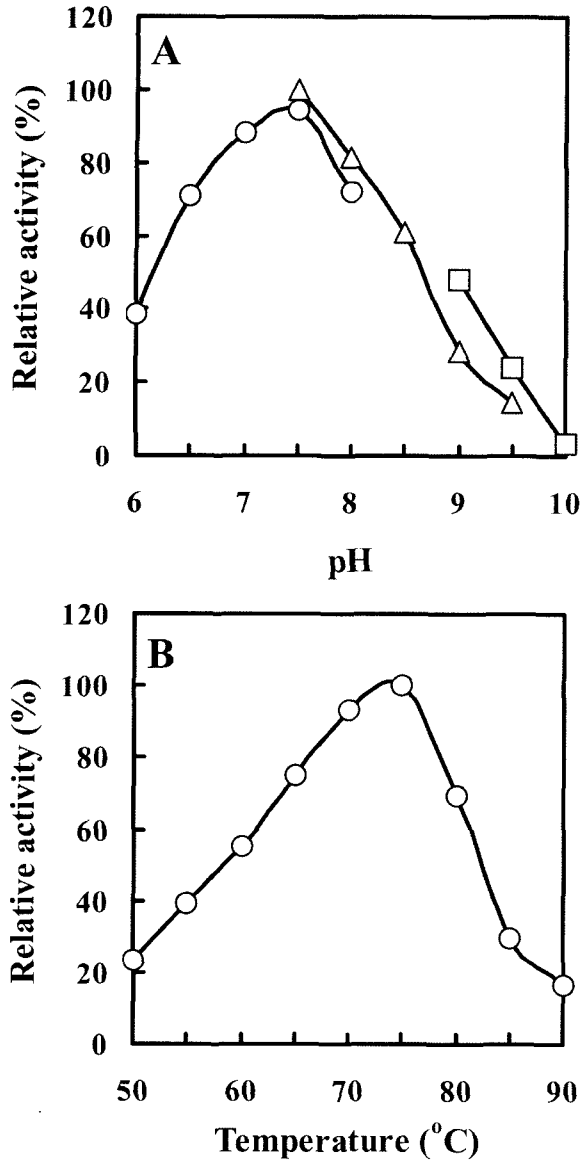

Fig. 3. The dependence of the *Par* DNA polymerase activity on pH and temperature.
(A) Effect of pH on the *Par* DNA polymerase activity: 25 mM Mops-NaOH (○), 25 mM Tricine-NaOH (△), and 25 mM Glycine-NaOH (□). **(B)** Effect of temperature on the *Par* DNA polymerase activity.

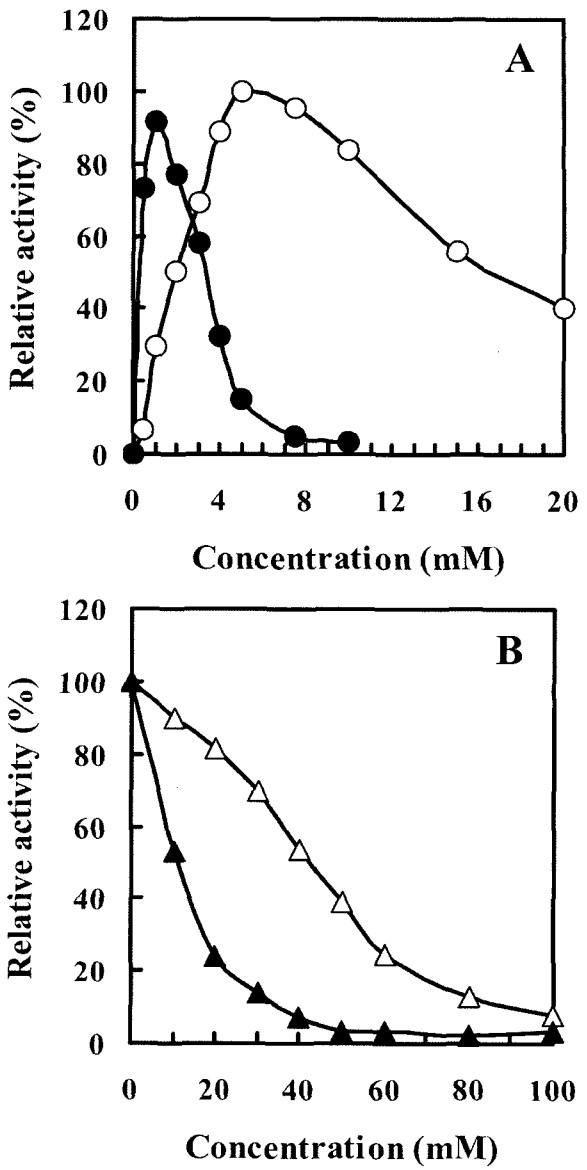

Fig. 4. Effects of cations on the *Par* DNA polymerase activity.
(A) Effects of divalent cations: Mg^{2+} (○) and Mn^{2+} (●). **(B)** Effects of monovalent cations: potassium ion (△) and ammonium ion (▲).

The effects of divalent cations on the *Par* DNA polymerase activity were examined with various concentrations of $MgCl_2$ and $MnCl_2$. The optimal Mg^{2+} concentration was 5 mM, and the optimal Mn^{2+} 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 Mg^{2+} 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 Mg^{2+} ion. Interestingly, the maximal activity of *Par* DNA polymerase by Mn^{2+} ion was similar to that by Mg^{2+} ion. In contrast, almost all of the DNA polymerases have been shown to have lower activity in the presence of Mn^{2+} than Mg^{2+} [10, 11]. The effect of Mn^{2+} 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 $(NH_4)_2SO_4$. 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

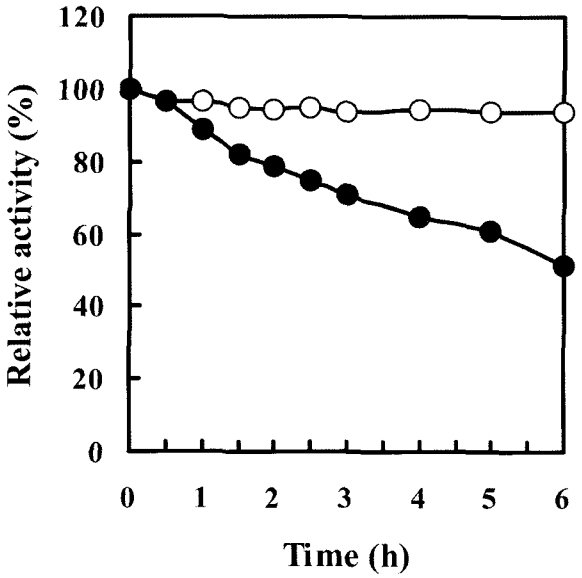

Fig. 5. Thermostability of *Par* DNA polymerase.
The purified *Par* DNA polymerase (20 μg/ml) was incubated at 75°C (○) and 95°C (●) in 25 mM Tricine-NaOH (pH 7.5), 2 mM $MgCl_2$, and 0.01% BSA. Aliquots of the mixture were removed at intervals up to 6 h and quenched on ice. The residual activity of the quenched samples was measured in the basic reaction mixture.

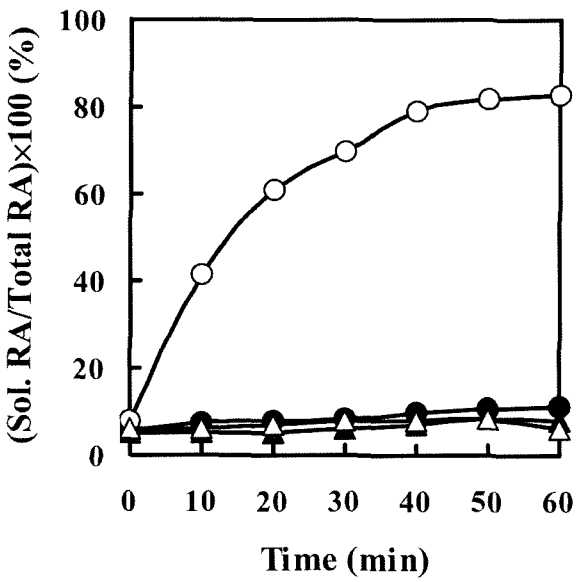

Fig. 6. Exonuclease activities of *Par* DNA polymerase.
3'→5' exonuclease activity was assayed in the absence (○) or presence (●) of dNTPs. 5'→3' exonuclease activity was assayed in the absence (△) or presence (▲) of dNTPs. Sol. RA, radioactivity of the supernatant solution after reaction; Total RA, total radioactivity of end-labeled DNA substrate used in the reaction.

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 ^{32}P-labeled product released from an end-labeled DNA substrate. When the substrate was labeled on its 3' end, about 83% of the ^{32}P was released in 1 h; however, when the substrate was labeled on its 5' end, no ^{32}P 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

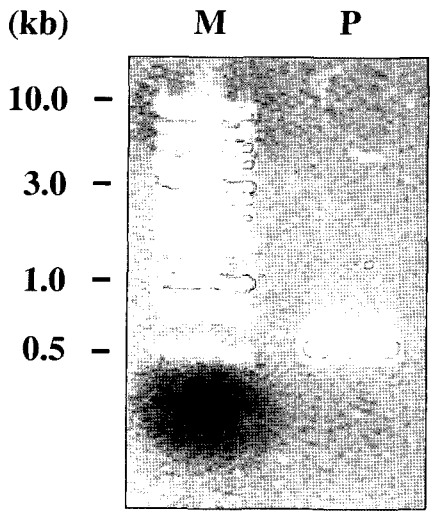

Fig. 7. PCR amplification with *Par* DNA polymerase.
The amplification of λ-phage DNA fragment was performed in a 50-μl reaction mixture containing 25 mM Tricine-NaOH (pH 7.5), 5 mM $MgCl_2$, and 0.01% BSA, as described in Materials and Methods. A 5-μl sample was subjected to electrophoresis on 1% agarose gel and stained with ethidium bromide. Lane M, DNA molecular size marker (sizes are shown on the left); lane P, the 500-bp amplified product.

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 $MgCl_2$, 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 archaeal 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

1. Bae, J.-D., Y.-J. Cho, D.-I. Kim, D.-S. Lee, and H.-J. Shin. 2003. Purification and biochemical characterization of recombinant alanine dehydrogenase from *Thermus caldophilus* GK24. *J. Microbiol. Biotechnol.* **13:** 628–631.
2. Barnes, W. M. 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proc. Natl. Acad. Sci. USA* **91:** 2216–2220.
3. Barns, S. M., C. F. Delwiche, J. D. Palmer, and N. R. Pace. 1996. Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *Proc. Natl. Acad. Sci. USA* **93:** 9188–9193.
4. Blanco, L., A. Bernad, M. A. Blasco, and M. Salas. 1991. A general structure for DNA-dependent DNA polymerases. *Gene* **100:** 27–38.
5. Braithwaite, D. K. and J. Ito. 1993. Compilation, alignment, and phylogenetic relationships of DNA polymerases. *Nucleic Acids Res.* **21:** 787–802.
6. Brown, J. R. and W. F. Doolittle. 1995. Root of the universal tree of life based on ancient aminoacyl-tRNA synthetase gene duplications. *Proc. Natl. Acad. Sci. USA* **92:** 2441–2445.
7. Cann, I. K. O., S. Ishino, N. Nomura, Y. Sako, and Y. Ishino. 1999. Two family B DNA polymerases from *Aeropyrum pernix*, an aerobic hyperthermophilic crenarchaeote. *J. Bacteriol.* **181:** 5984–5992.
8. Cann, I. K. O. and Y. Ishino. 1999. Archaeal DNA replication: identifying the pieces to solve a puzzle. *Genetics* **152:** 1249–1267.
9. Chien, A., D. B. Edgar, and J. M. Trela. 1976. Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *J. Bacteriol.* **127:** 1550–1557.
10. Choi, J. J., S. E. Jung, H.-K. Kim, and S.-T. Kwon. 1999. Purification and properties of *Thermus filiformis* DNA polymerase expressed in *Escherichia coli*. *Biotechnol. Appl. Biochem.* **30:** 19–25.
11. Choi, J. J. and S.-T. Kwon. 2004. Cloning, expression, and characterization of DNA polymerase from hyperthermophilic bacterium *Aquifex pyrophilus*. *J. Microbiol. Biotechnol.* **14:** 1022–1030.
12. Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* **16:** 10881–10890.
13. Dennis, P. P. 1997. Ancient ciphers: Translation in Archaea. *Cell* **89:** 1007–1010.
14. Erlich, H. A. 1989. *PCR Technology: Principles and Applications for DNA Amplification*. Stockton Press, New York, U.S.A.
15. Fitz-Gibbon, S. T., H. Ladner, U.-J. Kim, K. O. Stetter, M. I. Simon, and J. H. Miller. 2002. Genome sequence of the

hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*. *Proc. Natl. Acad. Sci. USA* **99:** 984–989.
16. Hanahan, D. and M. Meselson. 1980. Plasmid screening at high colony density. *Gene* **10:** 63–67.
17. Hoe, H.-S., S.-K. Lee, D.-S. Lee, and S.-T. Kwon. 2003. Cloning, analysis, and expression of the gene for thermostable polyphosphate kinase of *Thermus caldophilus* GK24 and properties of the recombinant enzyme. *J. Microbiol. Biotechnol.* **13:** 139–145.
18. Huber, H., M. J. Hohn, R. Rachel, T. Fuchs, V. C. Wimmer, and K. O. Stetter. 2002. A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature* **417:** 63–67.
19. Huber, R., M. Sacher, A. Vollmann, H. Huber, and D. Rose. 2000. Respiration of arsenate and selenate by hyperthermophilic archaea. *Syst. Appl. Microbiol.* **23:** 305–314.
20. Jung, S. E., J. J. Choi, H. K. Kim, and S.-T. Kwon. 1997. Cloning and analysis of the DNA polymerase-encoding gene from *Thermus filiformis*. *Mol. Cells* **7:** 769–776.
21. Kähler, M. and G. Antranikian. 2000. Cloning and characterization of a family B DNA polymerase from the hyperthermophilic crenarchaeon *Pyrobaculum islandicum*. *J. Bacteriol.* **182:** 655–663.
22. Kornberg, A. and T. Baker. 1992. *DNA Replication*, 2nd Ed. Freeman and Company, New York, U.S.A.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
24. Lee, J.-H., Y.-D. Cho, J. J. Choi, Y.-J. Lee, H.-S. Hoe, H.-K. Kim, and S.-T. Kwon. 2003. High-level expression in *Escherichia coli* of alkaline phosphatase from *Thermus caldophilus* GK24 and purification of the recombinant enzyme. *J. Microbiol. Biotechnol.* **13:** 660–665.
25. Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
26. Lundberg, K. S., D. D. Shoemaker, M. W. W. Adams, J. M. Short, J. A. Sorge, and E. J. Mathur. 1991. High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. *Gene* **108:** 1–6.
27. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3:** 208–218.
28. Mattila, P., J. Korpela, T. Tenkanen, and K. Pitkanen. 1991. Fidelity of DNA synthesis by the *Thermococcus litoralis* DNA polymerase, an extremely heat stable enzyme with proofreading activity. *Nucleic Acids Res.* **19:** 4967–4973.
29. Nishioka, M., H. Mizuguchi, S. Fujiwara, S. Komatsubara, M. Kitabayashi, H. Uemura, M. Takagi, and T. Imanaka. 2001. Long and accurate PCR with a mixture of KOD DNA polymerase and its exonuclease deficient mutant enzyme. *J. Biotechnol.* **88:** 141–149.
30. Reiter, W., U. Hüdepohl, and W. Zillig. 1990. Mutational analysis of an archaebacterial promoter: Essential role of a TATA box for transcription efficiency and start-site selection *in vitro*. *Proc. Natl. Acad. Sci. USA* **87:** 9509–9513.
31. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487–491.
32. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, New York, U.S.A.
33. Sanger, F., A. R. Coulson, G. F. Hong, D. F. Hill, and G. B. Petersen. 1982. Nucleotide sequence of bacteriophage lambda DNA. *J. Mol. Biol.* **162:** 729–773.
34. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. *Experiments with Gene Fusions*. Cold Spring Harbor Laboratory Press, New York, U.S.A.
35. Studier, F. W. and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189:** 113–130.
36. Truniger, V., J. M. Lazaro, M. Salas, and L. Blanco. 1996. A DNA binding motif coordinating synthesis and degradation in proofreading DNA polymerases. *EMBO J.* **15:** 3430–3441.
37. Uemori, T., Y. Ishino, H. Doi, and I. Kato. 1995. The hyperthermophilic archaeon *Pyrodictium occultum* has two α-like DNA polymerases. *J. Bacteriol.* **177:** 2164–2177.
38. Uemori, T., Y. Ishino, H. Toh, K. Asada, and I. Kato. 1993. Organization and nucleotide sequence of the DNA polymerase gene from the archaeon *Pyrococcus furiosus*. *Nucleic Acids Res.* **21:** 259–265.
39. Woese, C. R., O. Kandler, and M. L. Wheelis. 1990. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl. Acad. Sci. USA* **87:** 4576–4579.