PCR performance of the highly thermostable proof-reading B-type DNA polymerase from *Pyrococcus abyssi*

Jacques Dietrich a, Philippe Schmitt b, Montserrat Zieger b, Brigitte Preve b, Jean-Luc Rolland a, Hassan Chaabihi b, Yannick Gueguen a,*. 

a Ifremer, Direction des Ressources Vivantes, France
b Qbiogene, Parc d’Innovation, P.O. Box 72, 67402 Illkirch, France

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Abstract

DNA polymerase from the archaeon *Pyrococcus abyssi* strain Orsay was expressed in *Escherichia coli*. The recombinant DNA polymerase (Pab) was purified to homogeneity by heat treatment followed by 5 steps of chromatography and characterized for PCR applications. Buffer optimization experiments indicated that Pab PCR performance and fidelity parameters were highest in the presence of 20 mM Tris·HCl, pH 9.0, 1.5 mM MgSO4, 25 mM KCl, 10 mM (NH₄)₂SO₄ and 40 μM of each dNTP. Under these conditions, the error rate was 0.66·10⁻⁶ mutations/nucleotide/duplication. Pab DNA polymerase, having a half life of 5 h at 100°C, was demonstrated to be highly thermostable in PCR conditions compared to commercial Taq and Pfu DNA polymerases. These characteristics enable Pab to be one of the most efficient thermostable DNA polymerases described, exhibiting very high accuracy compared to other available commercial DNA polymerases and robust thermostable activity. This new DNA polymerase is currently on the market under the name Isis DNA Polymerase® (Qbiogene Molecular Biology).

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1. Introduction

*Pyrococcus abyssi* is an anaerobic hyperthermophilic archaeon that belongs to the Thermococcales order within the euryarchaeal subdomain. It was isolated from hydrothermal vents at a depth of 2000 meters in the southwest Pacific Ocean and grows optimally around 100°C [1]. The complete genome sequence of this archaeon has been analyzed by the Genoscope (Evry, France) and provides a resource for study of archael replication machinery. Two genes encoding a family B (α-like) and a family D DNA polymerases from this organism have been previously cloned and expressed in *Escherichia coli* [2]. Furthermore, due to their use in DNA sequencing and polymerase chain reaction (PCR) applications, thermostable DNA polymerases are technically and economically important enzymes.

More than 50 DNA polymerase genes have been cloned from various organisms [3–5]. These include family A DNA polymerases from thermophilic and hyperthermophilic bacteria (*Thermus aquaticus*, *Thermotoga maritima*, *Bacillus stearothermophilus*), as well as family B DNA polymerases from archaea (*Thermococcus litoralis*, *Pyrococcus furiosus*, *Thermococcus fumicolans*, ..) [6]. *Taq* polymerase is one of the most frequently used DNA polymerases for PCR applications. However, *Taq* has the considerable drawback of a high error rate and the lack of the ability to synthesize products larger than 5 kb [4]. The 3’→5’ exonuclease activity associated with many polymerases enhances polymerase fidelity. For example, *Pfu* and *Vent* DNA polymerases, with their associated 3’→5’ exonuclease, are widely used in applications requiring high fidelity [7,8].

In this paper, we report the purification and the characterization of the recombinant family B DNA polymerase from *P. abyssi* in terms of biochemical, PCR performance and fidelity parameters. These results are compared with data obtained using commercial DNA polymerases and demonstrate that this highly thermostable DNA polymer-
ase is very attractive for PCR applications requiring accurate amplification.

2. Materials and methods

2.1. Organisms and growth conditions

*P. abyssi* (strain Orsay) was used in this study. The complete genome sequence of *P. abyssi* was determined at Genoscope (Evry, France). Sequences and annotations are available at http://www.genoscope.cns.fr/Pab/. *E. coli* BL21(DE3) pLysS, was used as a host strain for overexpressing Pab DNA polymerase. *E. coli* strain was grown in 2×YT medium in a rotary shaker at 37°C with kanamycin (final concentration of 34 μg ml⁻¹).

2.2. Expression and purification of Pab DNA polymerase

The recombinant plasmid pPOL1 containing *pol1* (accession number P77916) gene was used for transformation of *E. coli* BL21(DE3)pLysS as described previously [2]. Overnight culture of *E. coli* BL21(DE3) pLysS harboring pPOL1 was diluted 1:20 and grown until OD₆₀₀ reached 0.6. Six liters of culture was induced with 0.5 mM of isopropyl-D-thiogalactopyranoside (IPTG) for 16 h. Cells were harvested by centrifugation and stored at −80°C. The yield of cells was 55 g wet weight. Fifty grams of cell culture was thawed at room temperature with 200 ml of 20 mM Tris–HCl, pH 8, 10 mM NaCl, 2 mM MgCl₂, 1% Triton X-100 and 0.1 mM phenyl methyl sulfonyl fluoride). The cells were disrupted by sonication using a Branson sonifier B-30. Cell debris were removed by centrifugation (35 000 × g for 30 min at 4°C). The resulting supernatant was heated for 15 min at 75°C and the precipitated proteins were removed by further centrifugation (100 000 × g for 1 h at 4°C). The supernatant constituted the fraction I.

2.3. Pab DNA polymerase purification

All subsequent steps were performed at 4°C. Protein samples were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli [9]. Protein concentrations were determined by the Bradford assay, with bovine serum albumin as the standard [10]. Protein bands were visualized by staining with Coomassie brilliant blue R-250. Endonuclease and nickase activity were tested at 37°C and 70°C for 16 h at each purification step. Supercoiled pBR322 DNA (1 μg) was incubated with 1–5 μl of each fraction, using different DNA polymerase buffers in a 50-μl final volume. (1) Q Sepharose fast flow anion exchange column (Amersham Biosciences): Fraction I was applied to a 60-ml Q-Sepharose fast flow column equilibrated with buffer A (50 mM Tris–HCl, pH 8, 0.5 mM EGTA, 0.5 mM DTT, 0.2% Triton X-100 and 10% glycerol). Bound proteins were eluted by a linear gradient of NaCl (0–0.5 M) in buffer A. The fractions containing partially purified polymerase eluted between 0.04 M and 0.1 M (fraction II). (2) Heparin hyperD affinity column (Life Technology): Fraction II was loaded to a 25-ml heparin hyperD column equilibrated with buffer A containing 0.1 M NaCl. A linear gradient of NaCl (0.15–1 M) in buffer A was applied. Pab DNA polymerase eluted between 0.3 M and 0.4 M (fraction III). (3) Blue Sepharose 6 fast flow affinity column (Amersham Biosciences): Fraction III was dialyzed against buffer A containing 0.1 M NaCl and applied to a 20-ml blue Sepharose fast flow column. Elution was performed with a linear gradient of NaCl (0.1–1 M). The polymerase eluted between 0.3 and 0.4 M (fraction IV). (4) HiTrap SP cation exchange column (Amersham Biosciences): The column (10 ml) was equilibrated in buffer B (20 mM, pH 6.5, 0.5 mM EGTA, 0.5 mM DTT and 10% glycerol). Fraction IV was dialyzed against buffer B containing 50 mM KCl and loaded onto the column. Elution was performed with a linear gradient of KCl (0.04–0.5 M) and the polymerase eluted between 0.1 M and 0.2 M KCl (fraction V). (5) Phenyl Sepharose 6 fast flow low sub hydrophobic column (Amersham Biosciences): The 10-ml column was equilibrated in buffer C (0.1 M NaPO₄, pH 6, 1.2 M (NH₄)₂SO₄ and 0.5 mM DTT). The fraction V was adjusted to 1.2 M (NH₄)₂SO₄ and loaded onto the column. A linear (NH₄)₂SO₄ gradient (1.2–0 M) was performed and the polymerase eluted between 0.6 and 0 M (NH₄)₂SO₄. The more active DNA polymerase fraction were pooled, dialyzed against storage buffer (20 mM Tris–HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Nonidet P40, 50% glycerol) and stored at −20°C (fraction VI).

2.4. DNA polymerase and exonuclease activity assay

DNA polymerase activity was assayed by measuring the incorporation of [methyl-³H]dTMP into trichloroacetic acid insoluble material as described previously [2]. Associated exonuclease activities were assayed using previously described methods [2].

2.5. Polymerase chain reaction

PCR performance of Pab DNA polymerase was assayed using various DNA substrates and its properties were compared to the properties of commercial *Pfu* (Stratagene), *Tfu* (Qbiogene) and *Taq* (Qbiogene) DNA polymerases. A range of 1 to 2 units of the various DNA polymerases were used in a PCR assay to generate DNA fragments of 420 bp or 1 kb, using 10 ng of genomic (human β-globin gene) or 1 ng of plasmidic DNA, respectively, as template. Except when indicated, PCR amplifications with Pab were performed with the optimized Pab conditions.
buffer (20 mM Tris–HCl, pH 9.0, 25 mM KCl, 1.5 mM MgSO₄, 0.1% Tween, 0.1 mg ml⁻¹ BSA, 10 mM (NH₄)₂SO₄), and 40 μM of each dNTP was used. Non-blocked primers were used at 50 pmol per PCR test in a 50-μl volume reaction. PCR products were analyzed by 1% or 2% standard agarose gel electrophoresis. The molecular mass ladder used is a pBR322 plasmid digested with HaeIII and TaqI (Qbiogene). Except where indicated, PCR amplifications with commercial DNA polymerases were performed in the presence of buffers supplied by manufacturers.

2.6. P53-based PCR fidelity assay

Error rates generated by Pab DNA polymerase were measured according to the previously described method of Flaman et al. [11]. Briefly, yeast cells were directly transformed with unpurified PCR products from p53 cDNA cloned into a linearized expression vector by homologous recombination in vivo. A 1.1-kb fragment containing the complete wild-type p53 open reading frame was amplified by using DNA polymerases. Mutations were scored according to the ability of the p53 product to activate transcription of an ADE2 reporter gene. The error rate per nucleotide and per cycle (E) was calculated using the equation $E = \frac{\text{mut}}{d \times \text{del} \times 100}$, where mut is the percentage of mutated ade2 (-) measured, d is the amount of effective amplified fragment doublings, and del is the effective target size (542 bp) of the complete p53. d is expressed by the equation $d = \log \frac{X}{\log 2}$, X being the amount of PCR product versus the amount of effective input template. PCR assays were carried out to amplify 20 pg of input DNA, with various DNA polymerase in a 50-μl volume. Different parameters were considered, such as the Mg²⁺ concentration (1.5–2–3 mM), dNTP concentration (40–100–200 μM each), KCl concentration (10–25–50 mM) and the presence of (NH₄)₂SO₄ (10 mM) having an inhibitory effect on 3'–5' exonuclease activity. The stoichiometry of Tris–HCl buffer, pH 9 (10–20 mM), was also investigated. Except when indicated, PCR amplifications with Pab were performed with the optimized Pab buffer (20 mM Tris–HCl, pH 9.0, 25 mM KCl, 1.5 mM MgSO₄, 0.1% Tween, 0.1 mg ml⁻¹ BSA, 10 mM (NH₄)₂SO₄). Each value is the mean of at least three assays.

3. Results and discussion

3.1. Expression and purification of Pab DNA polymerase

The 2313-bp pol1 gene encoding a family B DNA polymerase (Pab) was cloned into pET-26b+ under control of the T7 promoter. The Pab DNA polymerase was produced in recombinant form in E. coli BL21(DE3) pLysS cells after IPTG induction. Recombinant Pab was purified to homogeneity by a 6-step procedure: (1) incubation at high temperature that denatures most of the E. coli proteins, (2) Q Sepharose (removes nucleic acids and few proteins), (3) heparin hyperD (removes the majority of remaining contaminant proteins), (4) blue Sepharose (removes nonspecific nuclease activities at 37°C), (5) HiTrap SP (removes nickase activity at 37°C) and (6) phenyl Sepharose column chromatography. After this step, fraction VI contains DNA polymerase with high purity (>95%; Fig. 1), maximal specific activity (from 35 000 units mg⁻¹ at step 5, to 66 000 units mg⁻¹ at step 6) and no contaminating nickase or endonuclease activities from E. coli. It is possible that this step removes inhibitory compounds. All chromatographic steps are indicated to eliminate the maximum of contaminating proteins and DNA. Fraction VI will be used for the study of all molecular, biochemical characteristics and PCR assays.

SDS–PAGE analysis of the homogeneous proteins revealed a single band of about 85 kDa, corresponding to the calculated molecular mass of the pol1 product (Fig. 1). The predicted product is a protein of 771 amino acids, with a theoretical molecular mass of 84.8 kDa. Pab has strong amino acid similarities with already described thermophilic euryarchaeal DNA polymerases (Pfu, KOD, Deep Vent). Amino acids identities are higher than 74% compared with commercially available DNA polymerases [2]. Such close identity indicates that these polymerases are phylogenetically related and should have many common features.

3.2. Biochemical properties of Pab

Thermostability and pH dependency of Pab DNA polymerase were previously studied in a DNA polymerizing
activity test [2]. Pab was more active at pH 9 in glycine-NaOH buffer and pH 9.5 in CAPS buffer. Using Tris–HCl buffer, Pab retained more than 80% of its optimal activity between pH range 7–10 and appeared to possess an extended range of DNA polymerizing activity. Thermal stability of Pab was tested at different temperatures: 80°C, 90°C and 100°C. Pab DNA polymerase retained 75% of its activity after 48 h at 80°C and 50% of its activity after 12 h at 90°C and 5 h at 100°C. Pab is one of the most thermostable DNA polymerase when compared to Taq, Pfu [6], KOD (from Thermococcus kodakaraensis) [12] and Deep Vent (from Pyrococcus GB-D) DNA polymerases [6]. No 5′ → 3′ exonuclease activity was detected for Pab. Conversely, Pab was found to exhibit 3′ → 5′ exonuclease activity that confers proof-reading capability and enhances polymerase fidelity [2]. Proof-reading ability of DNA polymerases is directly related to the presence of three small sequence motifs named Exo I, Exo II and Exo III [5,8,13]. These domains could be identified within the deduced DNA polymerase sequence of P. abyssi. Such proof-reading DNA polymerase have to coordinate two opposite catalytic activities, polymerase and exonuclease functions, that were shown to reside in structurally distinct protein domains [14]. However, previous reports have suggested functional interdependence between the polymerase and exonuclease domains in family B DNA polymerases [8,14]. The relationship between the polymerase and the 3′ → 5′ exonuclease activity was described as a partitioning of the primer template between the two active sites [4].

3.3. PCR assays

The principal uses of thermostable polymerases are for in vitro amplification of DNA fragments and for determination of DNA sequence. PCR performances of Pab DNA polymerase were assayed using genomic and plasmid DNA templates. In addition, several types of PCR incubation buffers were analyzed. Results showed that, according to the type of DNA substrates used for PCR reactions, the ionic strength can affect the polymerase activity of Pab (Fig. 2). Compared to the optimized Pab incubation buffer (lane a), a decrease of the molarity of either Tris–HCl (lane c) or KCl (lane d) or an absence of (NH₄)₂SO₄ (lane f) strongly inhibits polymerase activity with a genomic DNA template (Fig. 2). Moreover, polymerase efficiency is sensitive to MgSO₄ concentration because an increase from 1.5 (lane a) to 3 mM (lane b) affects amplification. On the contrary, when plasmid DNA template is used for PCR reactions, reducing the ionic strength has a very low effect on the Pab amplification efficiency (data not shown). In addition, despite high 3′ → 5′ exonuclease activity, no modified primers were required and no premature digestion of the amplified products was noticed, even when final elongation was extended to 60 min (data not shown). Amplifications of a 4-kb fragment using mitochondrial human DNA template (10 ng) and a 7-kb fragment using lambda DNA template (10 ng) were performed using Pab DNA polymerase (data not shown).

The thermostability of Pab was also investigated in PCR amplifications and compared to commercial Taq and Pfu DNA polymerases. Results confirmed that Pab is one of the most thermostable proof-reading DNA polymerase described (Fig. 3). These results are interesting and indicate that denaturation time and temperature can both be increased if necessary in PCR reaction. This property can be useful for reading through difficult secondary structures or GC-rich regions of DNA template. Therefore, the robustness of Pab at high temperatures will permit the retention of more activity during PCR, giving more products at the end.

3.4. Fidelity assays

The use of high fidelity DNA polymerases in PCR is essential for reducing the introduction of amplification errors in PCR products that will be used in cloning and site directed mutagenesis [8]. We attempt here to improve
the fidelity of Pab by optimizing PCR reaction conditions. PCR errors rates were measured at varying concentrations of dNTPs, KCl and (NH4)2SO4. Replication fidelity of Pab was measured using a previously described assay [11] which measures the frequency of mutations introduced into the p53 target gene during PCR amplification. At a constant concentration of MgSO4 (1.5 mM), the error rate of Pab DNA polymerase decreases by reducing the dNTP concentration (Table 1). These results are consistent with those obtained by Cambon-Bonavita et al. [15] and Cline et al. [7] with Tfu and Pfu DNA polymerases and could be related to an increase of the mispair extension efficiency due to the diminution of the proof-reading ability of the polymerase [7]. Effectively, a number of factors are known to contribute to the overall fidelity of DNA polymerases [4,7]. One of these factors is the presence of a 3‘→5‘ exonuclease activity which can remove mispaired bases (proof-reading activity) up to five nucleotides after the misincorporation [4]. This importance of proof-reading activity has been demonstrated for Thermococcus litoralis (Vent polymerase) and Pfu DNA polymerases, which exhibits five-fold and seven- to 40-fold increase in error rate, respectively, when 3‘→5‘ exonuclease activities are inactivated [7,16].

An increase of MgSO4 concentration also alters Pab fidelity, as the error measured at 40 μM of each dNTP varied from 0.66×10⁻⁶ to 1.39×10⁻⁶, whereas the MgSO4 concentration varied from 1.5 to 3 mM (Table 1). In Table 2, the error rate of Pab was found to increase in the absence of (NH4)2SO4 and when Tris buffer molarity or KCl concentration decreases. A two-fold increase in error rate was observed when the KCl concentration varied from 25 to 10 mM. However, in absence of (NH4)2SO4 the loss of fidelity could be overcome by using a 50-mM KCl buffer, suggesting that accurate polymerization requires a minimum ionic strength. In conclusion, Pab DNA polymerase appears to be sensitive to buffer composition and its sensitivity to buffer conditions permits the modulation of fidelity from 0.66×10⁻⁶ to 4.7×10⁻⁶ through alteration of the MgSO4 and dNTP concentrations.

3.5. Optimized high fidelity PCR with Pab DNA polymerase

The combination of PCR and fidelity results has led to a hybrid buffer gathering the highest PCR efficiency and fidelity. The composition of the optimized Pab buffer is

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<th>Table 1</th>
<th>Effect of dNTPs and MgSO4 concentration on Pab DNA polymerase fidelity</th>
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<td>dNTP (μM)</td>
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Error rate was measured according to the method of Flaman as described in Materials and methods. Reactions were carried out in optimized Pab buffer with 3 units of Pab.

*Standard deviations were <5%, except for values with an asterisk (5% < standard deviation < 10%).

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<tr>
<th>Table 2</th>
<th>Effect of ionic strength on Pab DNA polymerase fidelity</th>
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<td>Tris molarity (mM)</td>
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Error rate was measured according to the method of Flaman as described in Materials and methods. Reactions were carried out in optimized Pab buffer with three units of Pab and 40 μM dNTP.

*Standard deviations were <5%, except for values with an asterisk (5% < standard deviation < 10%).

Fig. 3. Thermostability comparison of DNA polymerases in PCR reactions. PCR amplifications were carried out with 10 ng of genomic DNA (amplification of a 420-bp human β-globin gene fragment) with 50 pmol of unmodified primers, in the presence of 100 μM of each dNTP and 1 unit of the various DNA polymerases. The thermostability of Pab (is), Pfu from Stratagene (pf) and Taq from Qiogene (tq), used in their buffer, were compared using the following PCR programs. A: (5’ at 93°C) ×1—(1’ at 94°C, 1’ at 62°C, 1’15 at 72°C)×30. B: (5’ at 95°C) ×1—(2’ at 95°C, 1’ at 62°C, 1’15 at 72°C)×30. C: (5’ at 95°C) ×1—(1’ at 97°C, 1’ at 62°C, 1’15 at 72°C)×30. D: (5’ at 95°C) ×1—(1’ at 99°C, 1’ at 62°C, 1’15 at 72°C)×30. Molecular mass marker (MW) is pBR322 digested with HaeIII/TaqI.
20 mM Tris–HCl, pH 9.0, 25 mM KCl, 1.5 mM MgSO₄, 0.1% Tween, 0.1 mg ml⁻¹ BSA, 10 mM (NH₄)₂SO₄. Using this buffer, the replication fidelity of Pab was compared to commercial Taq, Tfu and Pfu DNA polymerases used in buffers supplied by manufacturers. Results indicate that Pab has a high fidelity equivalent to Pfu DNA polymerase and 40-times higher than Taq DNA polymerase (Fig. 4) with an error rate of 0.66 × 10⁻⁶ mutation frequency/bp/duplication according to the method of Flaman as described in Materials and methods. PCR was performed using 50 pmol of primers, in the presence of 40 μM of each dNTP and 3 units of the various DNA polymerases. Pab was used in the optimized Pab buffer, and Pfu (Stratagene), Tfu (Qbiogene) and Taq (Qbiogene) were used in their recommended buffers.

In conclusion, Pab DNA polymerase proves to be an easy enzyme to use in PCR applications and is available on the market under the name Isis DNA Polymerase™ (Qbiogene Molecular Biology). In optimum conditions, observed fidelity makes this highly thermostable DNA polymerase very attractive for applications requiring a strict limitation of errors in PCR.

References


