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Characterization of a family B DNA polymerase from *Thermococcus barophilus* Ch5 and its application for long and accurate PCR

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ABSTRACT

The family B DNA polymerase gene from the euryarchaeon *Thermococcus barophilus* Ch5 (*Tba*5) contains an open reading frame of 6198 base pairs that encodes 2065 amino acid residues. The gene is split by three inteins that must be spliced out to form the mature DNA polymerase. A *Tba*5 DNA polymerase gene without inteins (genetically intein-spliced) was expressed under the control of the pET-28b(+)T7*lac* promoter in *E. coli* Rosetta 2(DE3)pLysS cells. The molecular mass of the purified *Tba*5 DNA polymerase was about 90 kDa consistent with the 90,470 Da molecular mass calculated based on the 776 amino acid sequence. The optimal pH for *Tba*5 DNA polymerase activity was 7.5 and the optimal temperature was 70–75 °C. The enzyme possessed 3′ → 5′ exonuclease activity and was activated by magnesium ions. PCR amplification using *Tba*5 DNA polymerase enables high-yield for 1- to 6-kb target DNA products, while 8- to 10-kb target DNA products were amplified at low or inefficient levels. To simultaneously improve product yield and amplification fidelity, *Tba*5 plus DNA polymerase mixtures were constituted with various amounts of *Tba*5 DNA polymerase mixed with *Taq* DNA polymerase. The *Tba*5 plus DNA polymerase mixtures robustly amplified up to 25-kb λ DNA fragments. In addition, the PCR error rate of *Tba*5 plus3 and *Tba*5 plus4 mixtures were much lower than those of wild-type *Tba*5 DNA polymerase, *Pfu* DNA polymerase, *Taq* DNA polymerase, and *Pfu* plus DNA polymerase.

1. Introduction

Since the discovery of the first DNA polymerase (EC 2.7.7.7) in *Escherichia coli* by Lehman et al. in 1958 [1], a large number of DNA polymerase genes have been cloned and characterized from various organisms [2,3]. On the basis of amino acid sequence similarities, DNA polymerases can be classified into six families: A, B, C, D, X, and Y [4–6].

Thermostable DNA polymerases are widely applied in various fields with polymerase chain reaction (PCR) including molecular biology, biotechnology, and clinical diagnosis [7]. Especially, the most widely used thermostable DNA polymerase from *Thermus aquaticus* YT-1 has revolutionized modern molecular biology [8]. Although family A *Taq* DNA polymerase is the most commonly used DNA polymerase in PCR, this enzyme has two general drawbacks including length limitation of the product and a relatively high error frequency in the final product. Thus, the family B DNA polymerases from the hyperthermophilic Archaea have attracted considerable interest due to their remarkable thermal stability and their proofreading 3′ → 5′ exonuclease activity. Several family B DNA polymerases have been characterized and used for PCR, especially from species belonging to the *Thermococcales* order such as *Pyrococcus furiosus* [9], *Thermococcus litoralis* [10], *Thermococcus pacificus kodakarensis* [11], *Thermococcus peptonophilus* [12], *Thermococcus waiotapuensis* [13] and *Thermococcus pacificus* [14]. However, low processivity and slow PCR extension rates are drawbacks of family B enzymes. To overcome these problems, Barnes reported a novel method that utilized DNA polymerase mixtures containing both non-proofreading (Klentaq1) and proofreading (*Pfu*) DNA polymerases [15]. This mixture of enzymes allows for long and accurate PCR amplification of λ DNA targets [15]. Since the work of

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Barnes was reported, the enzyme mixtures have consisted of various archaeal DNA polymerases along with *Taq* DNA polymerase. This enzyme mixture is based on the following simple principle. DNA synthesis is catalyzed by means of 3′ → 5′ exonuclease-free *Taq* DNA polymerase at relatively high concentration and misincorporated nucleotides during an amplification reaction by *Taq* DNA polymerase is repaired and extended by a *Pfu* DNA polymerase with 3′ → 5′ exonuclease activity at low concentration [15]. These enzyme mixtures were observed to have enhanced PCR product size and fidelity better than *Taq* polymerase alone, whereas the majority of these enzyme mixtures were observed with reduced PCR fidelity compared to archaeal DNA polymerase alone [16–19].

Thermococcus barophilus Ch5 was isolated from a deep-sea hydrothermal field of the Mid-Atlantic Ridge [20], and whole-genome sequencing was conducted to search for thermostable enzymes. Analysis of the genome indicated that the strain possesses a family B-type DNA polymerase. This polymerase (*Tba*5 DNA polymerase) gene contains an open reading frame of 6198 bases that encodes 2065 amino acid residues. Interestingly, The gene is split by three inteins that must be spliced out to form the mature DNA polymerase. Up to now, the *Tba*5 DNA polymerase gene is one of the largest DNA polymerase genes among the known DNA polymerases.

In this study, the *Tba*5 DNA polymerase gene was cloned and expressed in *E. coli*. The recombinant DNA polymerase was purified, biochemically characterized, and successfully applied in PCR. We also report that a mixture of *Tba*5 and *Taq* DNA polymerases (named *Tba*5 plus) improved PCR performance for long and accurate PCR.

2. Material and methods

2.1. Strains and culture conditions

T. barophilus Ch5 was obtained from Dr. Tatyana Sokolova at the Winogradsky Research Center (Russia). *T. barophilus* Ch5 was isolated from a deep-sea hydrothermal field of the Mid-Atlantic Ridge [20]. YPS medium [21] was used to culture *T. barophilus* Ch5 for DNA manipulation and culture and strain maintenance were performed according to standard procedures [22]. *E. coli* strain DH5α was used for plasmid propagation for nucleotide sequencing. *E. coli* strain Rosetta 2(DE3) pLysS (stratagene, USA), which harbors the plasmid pET-28b(+) (Novagen, USA), was used for gene expression. *E. coli* strains were cultured in Luria–Bertani (LB) medium with 50 μg/ml kanamycin at 37 °C with vigorous shaking.

2.2. DNA manipulation and sequencing

DNA manipulation techniques were based on standard procedures, as described by Sambrook and Russell [23]. Genomic DNA of *T. barophilus* Ch5 was isolated using a standard procedure [22]. Restriction enzymes were purchased from TaKaRa (Japan). Small-scale preparation of plasmid DNA from *E. coli* cells was performed using a MEGAquick-spin kit (iNtRON Biotechnology, Korea). DNA sequencing was conducted using a BigDye terminator kit (PE Applied Biosystems, USA).

2.3. Cloning of the *Tba*5 DNA polymerase gene

The three intein-coding sequences of the *Tba*5 DNA polymerase gene are split by four exteins. To express the intein-spliced gene encoding the mature form of the *Tba*5 DNA polymerase, we amplified the four separate DNA polymerase coding regions (extein coding regions 1, 2, 3 and 4) using overlapping PCR (Fig. 1). Each primer contained a region that was complementary to the end of the neighboring extein sequence as follows. The regions encoding the N-terminal extein1 portion (sense primer, *Tba*5 Ex1F [5′-NNNNNCATATG ATTCTCGATACTGACTACATCACG-3′] and antisense primer, *Tba*5 Ex1R [5′-GGATACAGAGACCTAAAATCTAAATAAACAATGTTCT-3′]), mid-extein2 portion (sense primer, *Tba*5 Ex2F [5′-TAGATTTTAGGTCTCTGTATCCTTCAATAATCATC-3′] and antisense primer, *Tba*5 Ex2R [5′-CGTAGAAGCTGTTCGCCAAAAGCTTCACAGC-3′]), mid-extein3 portion (sense primer, *Tba*5 Ex3F [5′-TTTGGCGAACAGCTTCTACGGTTATATGGGGT-3′] and antisense primer, *Tba*5 Ex3R [5′-CCATCTGTGTCGGCATATAGAACTTTAAACC-3′]), and the C-terminal extein4 portion (sense primer, *Tba*5 Ex4F [5′-ATGCCGACACAGATGGACTCTATGCAACAAT-3′] and antisense primer, *Tba*5 Ex4R [5′-NNNNNNNGCGGCCGCCTTCCTCACATTCAGCCAGGCTC-3′]) were amplified separately. The *Tba*5 Ex1F and *Tba*5 Ex4R primers contained *Nde*I and *Not*I recognition sites, respectively (shown underlined). PCR reactions were performed in 50 μl total volume containing *Pfu* DNA polymerase buffer, 0.2 mM dNTPs, 20 pmole of each primer, 100 ng *T. barophilus* Ch5 genomic DNA, and 1.25 U *Pfu* DNA polymerase (Promega, USA). PCR reactions included parameters of 3 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 57 °C, and 1 min at 72 °C with an additional extension of 5 min at 72 °C using a MyGenie 32 thermal block (Bioneer, Korea). Each amplified PCR product (EX1, EX2, EX3 and EX4) was purified from 0.8% agarose gels using the MEGAquick-spin kit (iNtRON Biotechnology, Korea). Then, fragments EX1 + EX2 and EX3 + EX4 were allowed to hybridize by means of these overlapping regions. Then, the fragments EX1 + EX2 and EX3 + EX4 were used for consecutive PCR reactions using the *Tba*5 Ex1F + *Tba*5 Ex2R and *Tba*5 Ex3F + *Tba*5 Ex4R primers, respectively. These fusion fragments EX1-EX2 and EX3-EX4 fragments were purified as described above. These fragments was connected together in the same manner as described above with a final PCR reaction, resulting in the *Tba*5 DNA polymerase gene (fusion fragment EX1-EX2-EX3-EX4) lacking inteins. This modified *Tba*5 DNA polymerase gene of 2331 bp was digested with *Nde*I and *Not*I and cloned into pET-28b(+) forming the recombinant plasmid pTBA5.

2.4. Expression assay and purification of *Tba*5 DNA polymerase

E. coli BL21-CodonPlus(DE3), BL21-CodonPlus(DE3)-RIL, and Rosetta 2(DE3) pLysS (Stratagene, USA) cells each were transformed with the pTBA5 expression plasmid. Strains harboring the pTBA5 expression plasmid were grown at 37 °C in LB broth containing 50 μg/ml of kanamycin and chloramphenicol. Overexpression of the *Tba*5 DNA polymerase gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.3 mM at mid-exponential growth phase (OD_{600}, 0.6), followed by an additional 20 h of growth at 25 °C. The cells were harvested by centrifugation (6000 rpm, 4 °C, 20 min) and resuspended in buffer A (20 mM Tris–HCl buffer, pH 7.4, containing 0.3 M NaCl and 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted by sonication, cellular debris was removed by centrifugation at 13,000 rpm, at 4 °C for 15 min, and the supernatant was heat-treated for 30 min at 85 °C and centrifuged. The resulting supernatant was applied to 10% SDS-PAGE to assess protein production level. The quantity of *Tba*5 DNA polymerase bands was determined using the Quantity One program (Bio-Rad, USA).

The purification of *Tba*5 DNA polymerase was performed as previously described with slight modification [24]. The induced *E. coli* strain Rosetta2 (DE3) pLysS cells harboring pTBA5 were resuspended in 4 × *Taq* storage buffer (20 mM Tris–HCl, pH 8.0, 10 mM dithiothreitol, 0.1 mM EDTA, 100 mM KCl, 0.5% Nonidet P40 and 0.5% Tween 20) to one-twentieth. The suspension was incubated in a boiling-water bath for 10 min (5 min per cycle). After boiling lysis, the cell debris and denatured proteins were removed from the lysate by centrifugation at 13,000 rpm, at 4 °C for 20 min.